UNIVERSITÀ DEGLI STUDI DELLA TUSCIA

DIPARTIMENTO DI ECOLOGIA E SVILUPPO ECONOMICO SOSTENIBILE

DOTTORATO DI RICERCA IN ECOLOGIA E GESTIONE DELLE RISORSE BIOLOGICHE (XVIII CICLO)

TESI DI DOTTORATO:

FILOGEOGRAFIA COMPARATA DI ALCUNI ANFIBI ITALIANI: IMPLICAZIONI PER LA CONSERVAZIONE

DOTTORANDO
DR. DANIELE CANESTRELLI

COORDINATORE E TUTOR
PROF. GIUSEPPE NASCETTI

ANNO ACCADEMICO 2005-2006

A mia moglie Paola e mia figlia Alice,
le mie buone notizie ogni mattina,
i casi di studio che preferisco per le mie ricerche intorno alla diversità.

INDICE

PREMESSA

Questa tesi si basa sull'analisi dei pattern di diversità genetica in alcune specie di anfibi italiani. Nel capitolo 1 vengono presentate le tematiche e gli obiettivi generali che hanno ispirato lo studio. Per ognuna delle specie studiate i risultati ottenuti sono stati elaborati, discussi ed inviati a riviste scientifiche peer-reviewed per la valutazione e l'eventuale pubblicazione. Ciascuno dei capitoli da 2 a 5 è in ogni sua parte conforme al manoscritto inviato ad una rivista, ad eccezione della letteratura citata che viene presentata in forma cumulativa nell'ultima sezione della tesi. Infine, nel capitolo 6 viene presentata una discussione generale dei risultati ottenuti ed una analisi della loro rilevanza ai fini degli obiettivi generali dello studio.

1. INTRODUZIONE

La Filogeografia è la disciplina che si occupa dei principi e dei processi che hanno determinato l'attuale distribuzione geografica delle linee genealogiche dei geni, all'interno delle specie e tra specie strettamente affini (Avise, 1987). Nonostante la sua origine recente, le ricerche svolte in quest'ambito hanno prodotto una letteratura scientifica straordinariamente copiosa (Avise, 2000). L'interesse suscitato da questa disciplina nasce dalla possibilità che essa offre di far luce sui processi storici che hanno concorso a strutturare l'attuale distribuzione geografica della variabilità genetica, ma anche sul possibile ruolo di fattori recenti, di origine antropica, nonché sui processi evolutivi a livello di popolazioni, quali pattern di dispersione, passate frammentazioni e/o fluttuazioni demografiche, ecc. (Bermingham & Moritz, 1998; Avise, 2000; Emerson & Hewitt, 2005). Tra i suoi sviluppi certamente più interessanti vi è la Filogeografia Comparata, ossia lo studio dei pattern di concordanza (e discordanza) filogeografica tra taxa aventi distribuzione geografica sovrapposta. La ricerca di pattern comuni nella distribuzione geografica delle specie, è stata al centro dell'attenzione della scienza sin dalla nascita della Biogeografia come disciplina (Wallace, 1869). L'utilizzo di metodiche di genetica biochimica e molecolare e lo sviluppo di studi di filogeografia comparata sta però dando alla biogeografia nuove spinte, consentendole tra l'altro di indagare questioni eco-evolutive classiche, su scale spaziali e temporali più piccole di quanto non fosse stato possibile in precedenza (Arbogast & Kenagy, 2001; Zink, 2002). Questo approccio sta permettendo infatti di individuare eventi criptici di vicarianza che hanno svolto un ruolo determinante nello struttuare le comunità biotiche, di far luce sul ruolo della geografia nei processi di speciazione, nonchè di studiare l'associazione tra cicli climatici e distribuzione geografica delle specie e della variazione genetica all'interno di esse (Bernatchez & Wilson, 1998; Bermingham & Moritz, 1998; Zink, 2002). Esso si sta inoltre rivelando uno strumento particolarmente utile alla pianificazione delle strategie di conservazione di taxa minacciati dal crescente impatto antropico sugli ecosistemi, perchè in grado di fornire informazioni circa l'esistenza di aree

geografiche evolutivamente divergenti e di hotspot di biodiversità genetica (Moritz & Faith, 1998; Avise, 2004).

Nel presente lavoro viene utilizzato un approccio basato sulla filogeografia comparata, per indagare il ruolo di alcuni importanti processi storico-evolutivi nel determinare i pattern di diversità genetica in taxa della penisola italiana, e per fornire informazioni utili alla pianificazione degli interventi di conservazione della batracofauna. Di seguito i due obiettivi verranno introdotti e presentati separatamente.

1.1 OSCILLAZIONI CLIMATICHE DEL QUATERNARIO E PATTERN DI DIVERSITÀ GENETICA

Tra i fattori storici che hanno maggiormente contribuito a strutturare l'attuale distribuzione geografica delle specie e della variazione genetica all'interno di esse, vi sono le oscillazioni climatiche del Quaternario (Webb & Bartlein, 1992). L'entità e le modalità delle modificazioni indotte da tali eventi è assai diversa nelle diverse regioni del pianeta, particolarmente in dipendenza della posizione e distanza rispetto all'equatore e alle masse oceaniche, ed all'orientamento delle principali catene montuose (Hewitt, 2004a & 2004b e riferimenti all'interno). In Europa, durante le fasi pleniglaciali la calotta si estendeva a sud fino al 52° parallelo, gran parte della porzione centrale del continente era occupata da tundra e steppe fredde, ed il generale abbassamento del livello del mare di molte decine di metri determinò un arretramento della linea di costa che in alcuni casi, come in Adriatico, riguardò centinaia di chilometri (Van Andel & Tzedakis, 1996). Evidenze fossili, palinologiche e più recentemente genetiche, indicano che numerose specie animali e vegetali sopravvissero alle avverse condizioni climatiche vigenti durante queste fasi rifugiandosi prevalentemente nelle penisole meridionali iberica, italiana, balcanica e in Caucaso (ad es. Hewitt, 2000; ma vedi anche Stewart & Lister, 2001 and Deffontaine *et al.*, 2005). La condizione di allopatria avrebbe poi portato al lento accumulo di divergenza tra popolazioni residenti in distinti rifugi glaciali. Al

ripristino di condizioni climatiche più favorevoli, durante le fasi interglaciali, gli habitat settentrionali sarebbero stati ricolonizzati a partire dalle aree di rifugio, con conseguente formazione di zone di contatto secondario e di ibridazione tra linee evolutive differenziatesi appunto in rifugi diversi. Il maggior contributo a tale processo di ricolonizzazione sarebbe venuto dalle popolazioni del rifugio balcanico, mentre per effetto dell'orientamento geografico dell'arco alpino e dei Pirenei, minore sarebbe stato il contributo dalla penisola iberica e soprattutto da quella italiana. Una delle principali implicazioni di questo scenario generale è il pattern così detto di "southern richness, northern purity" (Hewitt, 1996, 1999 & 2000), ossia la maggior variabilità genetica riscontrata in popolazioni meridionali (quelle cioè situate nelle aree ipotizzate di rifugio) rispetto a quelle settentrionali. Tale maggior diversità è stata spesso attribuita alla prolungata stabilità demografica che avrebbe caratterizzato queste popolazioni, rispetto a quelle più settentrionali fondate attraverso un processo (l'espansione verso nord dell'areale) implicante successivi colli di bottiglia (Hewitt, 1996 & 2000; ma vedi Bilton *et al.*, 1998; Austerlitz *et al.*, 2000; Petit *et al.*, 2003). Più di recente, il gran numero di filogeografie publicate su taxa della penisola Iberica ha permesso di accostare a questo scenario classico, che implica l'esistenza nelle penisole meridionali di popolazioni grandi e demograficamente stabili per lunghi periodi (di qui in avanti definito scenario P.D.S.), uno scenario in parte alternativo definito di "refugia-within-refugia" (di qui in avanti R.F.R.; Sanz *et al.*, 2000; Guillame *et al.*, 2000; Gomez & Lunt, 2004). Esso prevede che la maggior diversità all'interno della penisola iberica sia da attribuire ad una significativa strutturazione geografica delle popolazioni imputabile almeno in parte all'esistenza di più aree di rifugio, o di fattori comunque in grado di determinare differenziamento allopatrico all'interno di questa penisola. Questa ipotesi, supportata da un crescente numero di evidenze (Jaarola & Searle, 2004; Vila *et al.*, 2005; per una rassegna v.d. Gomez & Lunt, 2004), ha diverse implicazioni. Anzitutto, per effetto della frammentazione, il processo di perdita di variabilità genetica durante la ricolonizzazione degli habitat settentrionali avrebbe avuto luogo a partire da un subset del pool genico originario, e sarebbe perciò stato più drastico. Inoltre, a questi eventi di micro-allopatria avrebbero potuto seguire fenomeni di contatto secondario, il cui ruolo nella formazione degli attuali pattern di

distribuzione della diversità e dei livelli di variabilità genetica delle popolazioni all'interno della penisola potrebbe essere stato fino ad oggi sottostimato.

Anche per quanto riguarda la penisola italiana, uno scenario di P.D.S. è stato ritenuto un plausibile tratto comune della storia di molti taxa, sia da quegl'autori che ne hanno enfatizzato il ruolo come rifugio glaciale e sorgente per le successive ricolonizzazioni degli habitat settentrionali, sia da coloro i quali ne favoriscono una visione come area ad elevata endemicità ma non come sorgente per le ricolonizzazioni (Hewitt, 1996; Bilton *et al.*, 1998; Petit *et al.*, 2003). La recente letteratura relativa a casi di studio dalla penisola iberica suggerisce tuttavia la possibilità che altri processi evolutivi abbiano contribuito a generare tale pattern. Primo obiettivo dello studio che viene qui proposto è dunque quello di valutare quale sia stato il contributo relativo degli scenari P.D.S. e R.F.R. nel determinare l'attuale distribuzione geografica della variazione genetica in taxa della penisola italiana. A tal fine si è scelto di concentrare l'attenzione sulla fauna anfibia, ed in particolare su tre specie di anfibi endemici italiani: *Salamandrina terdigitata*, *Hyla intermedia*, *Bombina pachypus*. Per ciascuna di esse verranno presentate analisi della struttura genetica delle popolazioni, studiata mediante l'utilizzo di marcatori sia nucleari (allozimi) che mitocondriali. I risultati ottenuti verranno valutati sia separatamente per ciascuna delle specie, che comparandoli tra loro e con altri casi di studio al fine di apprezzarne la generalizzabilità.

Alcuni tratti della biologia degli anfibi li hanno resi ormai da lungo tempo organismi molto apprezzati per studi di genetica delle popolazioni. In particolare, la loro generale tendenza ad una modesta vagilità e spiccata filopatria ne determina una scarsa abilità nell'oltrepassare barriere geografiche costituite da habitat sfavorevoli anche di modesta entità (e.g. Austin *et al.*, 2002). Ne consegue che essi siano organismi ideali per lo studio dell'influenza dei diversi fenomeni storici sui pattern di diversità genetica all'interno e tra le popolazioni. Inoltre, anche in virtù delle caratteristiche poc'anzi ricordate, è lecito supporre che specie con caratteristiche ecologiche diverse potrebbero aver risposto in modi diversi alle medesime vicende storiche, ad es. paleoclimatiche (vedi anche Avise, 2000). Perciò, essendo il primo obiettivo di questo studio quello di evidenziare il contributo relativo di processi evolutivi alternativi (P.D.S. vs. R.F.R.), piuttosto che non l'effetto di passati eventi

storici su un particolare ambiente e le specie ad esso legate, la scelta di studiare specie tra loro diverse in quanto a scelta dell'habitat (vedi paragrafo 1.3) è sembrata la strategia più appropriata. Considerato che le specie di anfibi qui studiate sono anche le prime in Italia peninsulare per le quali saranno disponibili dati da marcatori sia nucleari che mitocondriali, questa scelta consentirà inoltre di estendere le possibilità di comparazione dei risultati ottenuti ad un maggior numero di casi.

1.2 CONSERVAZIONE DEGLI ANFIBI IN ITALIA

Nel contesto della generale crisi della biodiversità, particolare attenzione sta ricevendo da ormai più di quindici anni il Declino Globale degli Anfibi, ossia il crollo demografico e l'estesa perdita di popolazioni che si sta verificando praticamente in tutto il pianeta a carico di questa classe di vertebrati. La World Conservation Union (IUCN) ha recentemente pubblicato il Global Amphibian Assessment, concludendo che almeno il 43% delle 5743 specie note di anfibi ha popolazioni in declino, ed il 32% è minacciata di estinzione. Questa percentuale è assai elevata, soprattutto se confrontata con quella relativa ad uccelli e mammiferi (12% e 23% rispettivamente; IUCN, 2004), due gruppi il cui stato di conservazione è universalmente considerato preoccupante. Un ulteriore elemento di preoccupazione deriva dal fatto che gli anfibi sono considerati ottimi indicatori dello stato di conservazione degli ambienti naturali (Vitt *et al.*, 1990; Blaustein & Wake, 1990; Barinaga, 1990; Blaustein & Wake, 1995; Beebee, 1996; Blaustein & Johnson, 2003), sicché un loro declino a livello globale rappresenta certamente un allarmante segnale circa l'attuale condizione degli ecosistemi (Baringa, 1990; Blaustein & Wake, 1995). Tra le maggiori cause del fenomeno vi sono certamente la distruzione e le gravi alterazioni degli habitat naturali da parte dell'uomo, il recente sviluppo epidemico di alcune infezioni, l'introduzione di fauna alloctona, ecc… (per estese revisioni si vedano ad es. Alford & Richards, 1999 e Gardner, 2001). Tuttavia una cospicua mole di casi di declino si sono verificati all'interno di aree protette o comunque non direttamente interessate da attività umane (ad es. Lips, 1998; Stallard, 2001; Blaustein & Kiesecker, 2002). Ciò, se da un lato ha determinato notevole

preoccupazione per l'impossibilità di indicare cause semplici e dirette del fenomeno, dall'altro ha portato allo sviluppo di una gran quantità di ricerche e all'utilizzo dei più diversi strumenti di indagine attraverso i quali è stato possibile giungere all'individuazione di diverse cause complesse ed interazioni tra fattori (Blaustein & Kiesecker, 2002; Blaustein & Johnson, 2003).

Tra i diversi paesi dell'area paleartica occidentale, l'Italia può essere considerata di gran lunga quello a maggior ricchezza di specie di anfibi, oltre la metà delle quali endemiche (Borkin, 1999). Tuttavia, a causa della scarsità di indagini sulla consistenza delle popolazioni attuali e dell'assenza di una adeguata documentazione storica (Shaffer *et al.*, 1998; Andreone & Luiselli, 2000), quadri organici sulla situazione conservazionistica degli Anfibi italiani sono decisamente scarsi, e si basano per lo più su considerazioni di carattere qualitativo (ad es. Bruno, 1973 e 1983; Andreone & Luiselli, 2000). Inoltre, per quanto riguarda gli studi di monitoraggio svolti su singole popolazioni, particolarmente grave è da ritenersi la carenza di dati relativi a lunghi periodi di tempo. Infatti, come ormai accertato da numerosi autori (ad es. Pechmann *et al.*, 1991; Pechmann & Wilbur, 1994; Skelly *et al.,* 1999; Skelly *et al.,* 2003) studi a breve termine hanno scarsissimo valore a fini conservazionistici, non potendo discriminare tra gli eventi di effettivo declino delle popolazioni e le fluttuazioni demografiche naturali caratterizzanti le sottopopolazioni di un sistema a metapopolazione (Hansky, 1994; Hecnar & M'Closkey, 1996; Marsh, 2001).

Per quanto riguarda le singole specie, il maggior contributo alla conservazione degli anfibi italiani è certamente venuto da studi di genetica delle popolazioni. Basti ricordare la profonda revisione che tali studi hanno determinato nell'assetto tassonomico di quasi tutti i gruppi investigati, con l'individuazione di numerose specie criptiche, molte delle quali endemiche. Ricordiamo in proposito, tra gli altri, il caso della raganella italiana (Nascetti *et al.*, 1995), della rana appenninica (Picariello *et al.*, 1990), della salamandra di Lanza (Nascetti *et al.*, 1988), delle diverse specie appartenenti ai generi *Hydromantes* (Nascetti *et al.*, 1996) e *Discoglossus* (ad es. Lanza *et al.*, 1984 & 1987). Altri studi su specie diverse hanno poi contributo ad accertarne la struttura genetica ed i livelli di variabilità genetica delle popolazioni (ad es. Santucci *et al.*, 1996; Ragghianti & Wake, 1986; Scillitani &

Picariello, 2000). Tuttavia, molte specie non sono ancora state studiate e soprattutto, nei soli casi delle due salamandre alpine *Salamandra atra* e *Salamandra lanzai* sono attualmente disponibili dati circa la struttura e la variabilità genetica relativamente a marcatori sia nucleari sia mitocondriali (Nascetti *et al.*, 1988; Riberon *et al.*, 2001 & 2002). Quest'ultimo aspetto costituisce per gli anfibi italiani un elemento di grave anomalia nel contesto internazionale degli studi di genetica delle popolazioni e genetica della conservazione. In anni recenti si sono infatti andate accumulando evidenze sempre maggiori del fatto che l'utilizzo di un solo marcatore genetico può facilmente portare a conclusioni parziali od errate, e che per contro il confronto dei risultati ottenuti con marcatori aventi modalità di trasmissione ereditaria ed evoluzione diverse, possa costituire in molti casi l'unico strumento attraverso cui giungere ad una corretta documentazione dello stato delle popolazioni e soprattutto alla comprensione dei fenomeni storici che tale stato hanno maggiormente contribuito a determinare (vedi ad es. Piel & Nutt, 2000; Shaw, 2002).

Da quanto fin qui detto emerge come a tutt'oggi non esista un quadro d'insieme tale da costituire quella base scientifica fondamentale per una corretta pianificazione degli interventi di gestione delle specie e di prioritizzazione conservazionistica delle aree geografiche dove esse insistono.

Come sopra ricordato, gli studi di filogeografia e filogeografia comparata possono fornire informazioni di grande rilievo per la biologia della conservazione, in particolare consentendo l'identificazione di aree geografiche evolutivamente divergenti, su cui basare una zonazione "eco-evolutiva" (da contrapporsi all'attuale "politico-aministrativa") degli interventi di conservazione, nonché di possibili hotspot di diversità genetica verso cui dirigere prioritariamente tali interventi. Ciò individua il secondo obiettivo del presente lavoro, ossia quello di contribuire alla conservazione degli anfibi italiani fornendo il primo contributo utile proprio all'identificazione di tali aree.

LE SPECIE STUDIATE

Le specie oggetto di questo studio, lo ricordiamo, sono tre: la salamandrina dagli occhiali, la raganella italiana, l'ululone appenninico. Di seguito, ciascuna di esse viene presentata brevemente.

La salamandrina dagli occhiali, *Salamandrina terdigitata* (Lacépède, 1788), è una specie endemica italiana, diffusa lungo l'arco appenninico dalla Liguria alla Calabria. Essa è anche l'unica rappresentate del suo genere, che tuttavia doveva avere un tempo una diffusione più ampia come testimoniano reperti fossili trovati in Sardegna e Grecia (Lanza, 1988 e riferimenti all'interno). La distribuzione altitudinale di questa specie è abbastanza ampia, dal livello del mare ad oltre 1300 m s.l.m., sebbene sia più frequente nella fascia compresa tra i 200-900 m s.l.m. (e.g. Lanza, 1983; Mazzotti *et al.*, 1999; Corsetti & Angelini, 2000). L'habitat per l'ovideposizione è costituito prevalentemente da torrenti e ruscelli poco profondi e a corso lento, ma non di rado vengono utilizzati i più diversi tipi di ambienti umidi, quali piccoli fossi, pozze effimere, fontanili.

Per quanto riguarda l'inquadramento conservazionistico, la salamandrina dagli occhiali è citata negli Allegati II e IV della Direttiva Habitat (Direttiva 92/43/CEE) ed è inserita in Appendice II della Convenzione di Berna. Essa è inoltre protetta in Italia da leggi a carattere regionale. Tra le molteplici cause di preoccupazione conservazionistica vanno certamente annoverate la frammentazione degli habitat, nonchè l'inquinamento e le alterazioni strutturali dei siti riproduttivi (regimentazionedelle acque, captazione, ...) e delle aree ad essi associati, ad es. mediante esbosco (ad es. Corsetti & Angelini, 2000).

La raganella italiana, *Hyla intermedia* (Boulenger, 1882), è una specie endemica dell'Italia e della Sicilia, fino a pochi anni fa attribuita alla specie *H. arborea* e rivalutata quale buona specie in seguito a ricerche di genetica biochimica (Nascetti *et al.,* 1995). La raganella italiana è un anfibio che nei periodi di attività mostra abitudini spiccatamente arboricole. Attiva principalmente nelle prime ore notturne, predilige per la riproduzione acque ferme o debolmente correnti, stagni a disseccamento stagionale, piccoli laghi, ma anche risaie o cave in disuso. Sembra che la riproduzione possa avvenire anche in acque debolmente salmastre (Lanza, 1983)

oltre che in pozze di acqua sulfurea (Cucchiara *et al.*, 1996). Per quanto riguarda la distribuzione altitudinale la raganella italiana mostra una spiccata preferenza per le aree di pianura sia costiere che interne. Da quanto riportato negli atlanti erpetologici regionali sembra che l'80-90% delle popolazioni siano situate a quote inferiori ai 400 m s.l.m. (v.d. ad es. Venchi, 2000; Mazzotti *et al.*, 1999; Gasc *et al.*, 1997), sebbene soprattutto nelle porzione meridionale dell'areale esistano segnalazioni di piccole popolazioni situate al di sopra dei 1000 m s.l.m. (c.a. 1500 m s.l.m. presso i Monti della Laga, Ri).

Inserita in Appendice II della Convenzione di Berna (sotto *Hyla arborea*) e protetta in Italia da leggi regionali, mancano a tutt'oggi studi organici circa lo stato di conservazione di questa specie. Tuttavia è noto che nel corso dell'ultimo secolo essa è scomparsa da estese porzioni dell'areale. Basti citare il caso della Pianura Padana e dell'Agro Pontino, aree che fino circa a metà del secolo scorso la specie popolava con continuità e con popolazioni straordinariamente consistenti, e dove oggi essa risulta invece ampiamente frammentata. Tra le principali cause di minaccia vanno certamente ricordate la distruzione degli habitat riproduttivi, come le così dette zone umide minori, l'inquinamento e la frammentazione di quelli ancora esistenti, l'introduzione di fauna alloctona (soprattutto ittica) e la diffusione di pratiche agricole monocolturali (per più estese discussioni in proposito si vedano Canestrelli, 2002 e Scoccianti, 2001).

L'ululone appenninico, *Bombina pachypus* (Bonaparte, 1838), è un anfibio anuro endemico dell' Italia peninsulare, presente lungo tutto l'arco appenninico dalla Liguria alla punta della Calabria (Lanza, 1983). La sua presenza in Sicilia, pur segnalata per il settore nord-orientale (Bruno, 1970), attende ulteriori conferme. Specie prevalentemente diurna, i siti di riproduzione sono costituiti per lo più da pozze temporanee di piccole dimensioni, poco profonde e soleggiate. Dal punto di vista altitudinale, l'ululone appenninico predilige quote comprese tra 200 e 800 metri s.l.m. (Caputo *et al.*, 1985; Doria & Salvidio 1994; Mazzotti *et al.*, 1999; Sarrocco & Bologna, 2000), anche se occasionalmente lo si può ritrovare fino a 1800 m s.l.m., o al livello del mare (Lanza, 1983; Mazzotti *et al.*, 1999; Sarrocco & Bologna, 2000). Complessivamente dunque, sia dal punto di vista latitudinale sia altitudinale, la

distribuzione geografica di questa specie risulta ampiamente sovrapposta a quella della salamandrina dagli occhiali.

La specie (sotto *B. variegata*, della quale era fino a poco tempo fa considerata sottospecie) è inserita negli Allegati II e IV della Direttiva Habitat (Direttiva 92/43/CEE) ed è citata in Appendice II della Convenzione di Berna. Essa è inoltre protetta in Italia su base regionale. L'areale di distribuzione della *Bombina pachypus* risulta fortemente frammentato ed è stato più volte suggerito che diverse popolazioni stanno subendo declino (vedi ad es. Sarrocco & Bologna, 2000; Caputo *et al.*, 1985; Doria & Salvidio, 1994 e riferimenti all'interno). Inoltre diversi autori hanno sottolineato come le popolazioni esistenti siano spesso costituite da un numero esiguo di individui (ad es. Sarrocco & Bologna, 2000). Come per le specie precedenti, tra le cause di minaccia sono certamente da annoverare la distruzione degli habitat riproduttivi e la frammentazione di quelli ancora esistenti, anche a seguito dei cambiamenti nelle tecniche agricolturali. Inoltre per questa specie è stato segnalato il rinvenimento in popolazioni dell' Appennino settentrionale di individui affetti da *Batrachochytrium dendrobatidis* (Stagni *et al.*, 2002), un fungo patogeno che ha causato gravi fenomeni di declino in popolazioni di diverse specie di Anfibi da molte parti del pianeta (Daszak, & Hyatt, 2003).

2. GENETIC EVIDENCE FOR TWO DISTINCT SPECIES WITHIN THE ITALIAN ENDEMIC *Salamandrina terdigitata* LACÉPÈDE, 1788 (AMPHIBIA: URODELA: SALAMANDRIDAE).

DANIELE CANESTRELLI, FRANCESCA ZANGARI & GIUSEPPE NASCETTI.

ABSTRACT

Genetic variation in 12 populations of the Italian endemic spectacled salamander, *Salamandrina terdigitata* (Lacépède, 1788) was investigated through the analysis of 29 allozyme loci and partial sequences of two mitochondrial genes (cytochrome *b*, 629 bp and 12s rRNA, 444 bp). Both nuclear and mitochondrial markers fully agreed in identifying two well-differentiated population groups, one ranging from Tusco-Emilian Apennine to southern Latium, the other comprising populations from central Campania to Calabria. At the nuclear level, nine diagnostic and four highly differentiated loci led to an average genetic distance of D_{NEI}= 0.47 between both groups, while within them D_{NEI} ranged from 0.00 to 0.05. At the mitochondrial level two alternatively fixed haplogroups were found, with an average Kimura-2-parameter sequence divergence of 17% and 8% at the cytochrome *b* and 12s rRNA genes respectively. The observed genetic structure and the comparison between the levels of divergence recorded with respect to those reported for other salamanders, strongly suggest that two distinct species were so far included within *Salamandrina terdigitata*. The names *Salamandrina perspicillata* (Savi, 1821) and *S. terdigitata* are here proposed for the species from central and southern Italy respectively.

KEYWORDS: *Salamandrina terdigitata, Salamandrina perspicillata,* spectacled salamander, allozymes, mitochondrial genes, cytochrome *b*, 12s rRNA, molecular taxonomy.

2.1 INTRODUCTION

Since their emergence, biochemical and molecular techniques have allowed the study of the genetic structure of populations, providing evidence for the existence of cryptic biodiversity that was previously unsuspected. For amphibians, which are generally conservative in their morphological evolution (Cherty *et al.*, 1978; Hass *et al.*, 1995; Richards & Moore, 1996), the routine use of these tools has led to the identification of an astonishing number of morphologically "cryptic" species (e.g. Duellman, 1993; Nascetti *et al.*, 1996; Hanken, 1999; Frost, 2002), even in the well-studied European batrachofauna, as recently reviewed by Veith (1996) and Borkin (1999). In fact, the number of amphibian species recognized for the European area has almost doubled during the last four decades (Mertens & Wermuth, 1960; Frost, 2002), but it should be borne in mind that several species have not yet been investigated. Among these, *Salamandrina terdigitata* constitutes an interesting case study. Many aspects of its natural history are well known, it is of particular concern to Italian zoologists because it is the only Italian endemic terrestrial vertebrate genus (Lanza, 1988), and is protected by international and regional laws (it is listed in the Annexes II and IV of the EU Council Directive for the Conservation of Natural Habitats and of Wild Fauna and Flora). In spite of this, no studies have investigated its geographic variation and genetic population structure to date. This paper is therefore aimed at filling this gap.

The spectacled salamander, *Salamandrina terdigitata,* is a stream-breeding species endemic of peninsular Italy, mainly distributed on the western side of the Apennine chain from 200m to 900m a.s.l. (Lanza, 1983a; Mazzotti *et al.*, 1999; Corsetti & Angelini, 2000, see Figure 1). It is the only known representative of a genus which, according to the morphological and molecular analysis done by Titus & Larson (1995), is an ancient lineage that separated from other newt lineages very shortly after the split between newts and true salamanders. Fossil records revealed that its distribution was once wider than now, comprising at least Sardinia and Greece (Lanza, 1988 and references therein).

Since a growing body of evidence shows that the use of only one kind of marker (i.e. cytoplasmatic or nuclear) can lead to misconceptions about population

structure and history (e.g. Piel & Nutt, 2000; Shaw, 2002), we here provide data on the genetic population structure of the spectacled salamander assessed by means of allozyme electrophoresis (i.e. a set of nuclear markers) and partial sequences of two mitochondrial genes, cytochrome *b* and 12s rRNA. Finally, the taxonomic implications of our results will be also discussed.

2.2 MATERIALS AND METHODS

We collected 149 specimens of *Salamandrina terdigitata* from 12 populations covering almost the entire species range (Fig.1). The geographical origin of the samples studied and sample sizes are presented in Table 1. Each specimen was anaesthetized in the field with 3-aminobenzoic acid ethyl ester (MS222) following the protocol of Heyer *et al.* (1994) and tail-clipped (about 2 cm) before being released in the same place. Tail samples were transported to the laboratory in liquid nitrogen containers and stored at –80 °C until further analyses. In order to adjust technical procedures and score liver-active enzymes, five specimens from each sampling site were euthanasized with an excess of MS222, then samples of skeletal muscle and liver were obtained and stored at –80 °C.

2.2.1 ALLOZYMES

Tissues from each specimen were crushed in 0.1 ml of distilled water and adsorbed onto chromatography paper labels. Horizontal electrophoresis was carried out onto 10% starch gels. We studied electrophoretically 20 enzymes encoded by 29 presumptive loci (see Table 2 for description of systems and electrophoretic conditions). Isozymes were numbered in order of decreasing mobility from the most anodal one (*Ldh-1* and *Ldh-2* correspond to *Ldh-A* and *Ldh-B* respectively). Alleles at each locus were designated by their mobility (in mm, standardized conditions) relative to the most common one (*100*) in the reference population (Taverna, Calabria).

Allele frequencies and estimates of genetic variability (mean observed and expected heterozygosity, percentage of polymorphic loci, and average number of

alleles per locus) were calculated for each population using the software BIOSYS-2 (Swofford & Selander, 1999). Exact significance tests for Hardy-Weinberg equilibrium (HW) were conducted for each locus and sample, then the Bonferroni correction for multiple tests was applied (Rice, 1989).

Genetic distances between populations were calculated with Nei's (1972) standard genetic distance matrix, which was then used to build an UPGMA phenogram. 1000 bootstrap pseudoreplicates over loci were run to test the reliability of the UPGMA phenogram with the BOOTDIST option in BIOSYS-2. The consensus UPGMA was then obtained using the subroutines NEIGHBOR and CONSENSE in the software PHYLIP 3.5c (Felsenstein, 1993). The cophenetic correlation coefficient, which measures the correlation between distance values calculated during tree building and the observed distance, was also computed. In addition, a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was carried out in order to partition total genetic variance into covariance components due to differences within populations, among populations within groups and between groups, using ARLEQUIN 2.000 (Schneider *et al.*, 1999).

2.2.2 MITOCHONDRIAL DNA

Total genome DNA was extracted from frozen tissues following standard phenol/chloroform extraction protocol (Sambrook *et al.*, 1989). We amplified partial sequences of two mitochondrial genes: the cytochrome *b* and the ribosomal 12s rRNA (12s) via polymerase chain reaction (PCR). For both fragments PCRs were carried out in a final volume of 50 µl containing: 1 µl of genomic DNA, 30 mM KCl, 10 mM Tris HCl pH 8.3, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, and 2.5 units of Taq polymerase. PCR cycles for cytochrome *b* consisted of: 5 minutes of denaturation at 95°C, followed by 40 cycles including denaturation at 93°C for one minute, annealing at 45°C for 45 seconds and extension at 72°C for one minute and 30 seconds, followed by a final extension of 10 minutes at 72°C. PCR cycles for 12s were the same except for the annealing step (50°C for 45 seconds). We used the following PCR primers: MVZ15-L and MVZ16-H (cytochrome *b*, Moritz *et al.*, 1992), and 12SZ–L and 12SF–H (12s, Goebel *et al.,* 1999). The PCR products were visualized on 1% agarose gels containing 0.5 µg/ml

Ethidium Bromide for the staining under UV light. The purified PCR products were double-sequenced using the same PCR primers on an ABI3730 XL automatic DNA sequencer with the standard protocol of the BigDye Terminator v3.1 Cycle Sequencing Kit.

The sequencing chromatograms were analyzed with the program CHROMAS ver. 2.23 (Technelysium Pty Ltd, Australia). Alignments were produced with the software CLUSTALX 1.81 (Thompson *et al*, 1997). All haplotypes found were deposited in Genbank (Accession Numbers AY695901- AY695907).

We calculated the number of substitutions and genetic distances between haplotypes according to Kimura-2-parameter model (K2P; Kimura, 1980) as implemented in the software MEGA 2.1 (Kumar *et al.*, 2001). This widely-used model (K2P) was chosen because to compare our results with previous studies. Phylogenetic analyses were performed using Maximum Parsimony (MP) and Neighbor Joining (NJ) methods as implemented in PAUP* 4.0b10 (Swofford, 2003). Bootstrap resampling was performed on 1000 pseudoreplicates. Unweighted MP trees were obtained using the exhaustive search. *Salamandra lanzai* was used as outgroup (GenBank Accession Number AF356699; Riberon *et al.*, 2002).

2.3 RESULTS

2.3.1 ALLOZYMES

Thirteen out of twenty-nine loci analyzed (*G3pdh, Ldh2, Mdh1, Icdh1, 6Pgdh, G6pdh, Sod1, Aat2, Ck, Adk, PepB2, PepD1,* and *Pgm1*) were monomorphic in all populations surveyed. Allele frequencies at the sixteen polymorphic loci are presented in Table 3. None of the tests for HW equilibrium was significant after Bonferroni correction. Based on allele frequencies at polymorphic loci, the samples can be grouped into two well-differentiated groups, one comprising populations from central Italy (samples 1 to 6), and the other including those from southern Italy (samples 7 to 12). This subdivision is reflected by the two main clusters in the UPGMA phenogram showed in Figure 2 (cophenetic correlation coefficient CCC = 0.995). Nine loci (*Mdhp1, Mdhp2, Gapdh, Aat1, Alat, PepB1, PepD2, Tpi, Pgm2*)

were fully diagnostic between both groups of populations, while at the other four loci (*Ldh1, Icdh2, Mpi, Gpi*) distinct alleles were found at moderate to high frequencies in only one of these groups (see Table3). Pairwise values of Nei's (1972) genetic distances are presented in Table 4. Genetic distances between groups ranged from D_{NEI}=0.41 to D_{NEI}=0.52, with an average value of D_{NEI}=0.47 (standard deviation -SD- = 0.03), whereas within each of the two groups it varied from D_{NEI}=0.00 to D_{NEI}=0.05, with an average value of D_{NEI}=0.02 (SD = 0.02). Within the southern group, populations 11 and 12 (Calabria) were the most differentiated, with high bootstrap support (88%) in the UPGMA analysis and presenting an average genetic distance of D_{NEI}=0.03 (SD = 0.01) with respect to the other southern samples. When populations were grouped into the two main groups identified by the UPGMA analysis, the results of the AMOVA analysis (Table 5) indicated that up to 91 % of the total genetic variability found in our dataset can be attributed to differences among groups.

Estimates of genetic diversity are presented in Table 3. Expected heterozygosity varied from 0.00 to 0.08, with the highest values in populations from the central group (populations 1, 5 and 6). Within the southern group, the population from Amalfi completely lacks genetic diversity, and that from Serino was polymorphic at only one locus (*Ldh1*), with H_E = 0.01. Other measures of genetic variability (observed heterozygosity, percentage of polymorphic loci and average number of alleles per locus) exhibited the same geographical pattern as H_E.

2.3.2 MITOCHONDRIAL DNA

The amplified fragments of the cytochrome *b* gene yielded unambiguous sequences of 629 bp without indels, while 12s sequences varied in length from 436 to 444 bp.

For cytochrome *b*, five distinct haplotypes were defined by 95 variable positions (79 at third positions, 11 at first and five at second, including nine aminoacid replacements). The NJ analysis (Figure 3) identified two well-differentiated haplogroups, with an average K2P genetic divergence of 0.17 (SD = 0.02) and a geographical distribution that unequivocally reflects the pattern observed for the allozymes. The same topology was also obtained by MP (a single tree of 194

steps in length, consistency index CI = 1.00, retention index RI = 1.00, homoplasy index HI = 0.00). All southern specimens (samples 7 to 12) shared a single haplotype (s1), which was never found in samples from central Italy (samples 1 to 6). In the latter, four haplotypes (c1-c4) were found to differ in at most two nucleotide substitutions. Haplotypes c3 and c4 were both observed only once in the population from Percile, whereas c2 was found in a single specimen from Bagno di Romagna. All other specimens from the central-Italy population group shared the haplotype c1.

Nucleotide variation was less pronounced at the 12s fragment: only two distinct haplotypes with a K2P genetic distance of 0.08 were detected, one in each group of samples.

2.4 DISCUSSION

We observed a particularly significant pattern of genetic differentiation among the studied populations. In fact, both mitochondrial and nuclear markers fully coincide in delimiting two well-defined clusters of closely related samples, one comprising the samples from Tusco-Emilian Apennine to southern Latium (samples 1-6), and the other including those from central Campania to southern Calabria (samples 7-12). As evidenced by the AMOVA analysis, most of the genetic variation observed at allozymes (91 %) can be attributed to differences between both groups, with only a small amount of the total variation attributable to within-group variation.

Levels of genetic divergence were found to be unexpectedly high considering the restricted distribution of this species (it is an Apennine endemism [e.g. Lanza, 1983a]), as well as its apparent morphological homogeneity. The pattern of genetic divergence observed with allozymes (nine diagnostic loci, and four other that were highly differentiated at allele frequencies, leading to an average $D_{NEI} = 0.47$) resembles that found between several congeneric species of salamanders. For instance, Nascetti *et al.* (1996) found values of D_{NEI} ranging from 0.33 to 0.40 among species of *Hydromantes* from mainland Italy (*H. italicus*, *H. ambrosii* and *H.*

strinatii), whereas among species of *Hydromantes* from eastern Sardinia (*H. flavus*, *H. imperialis* and *H. supramontis*) the same authors (see also Lanza *et al.*, 1995) found D_{NEI} values ranging from 0.47 to 0.48. Similar values have been also reported for some species of the genus *Triturus* (e.g. Macgregor *et al.*, 1990). At the mitochondrial level, the values of K2P divergence observed within *S. terdigitata* (17 % for cytochrome *b* and 8 % for 12s) exceed those reported in the literature for other congeneric species of salamanders. For instance, Riberon *et al.* (2001) reported K2P values for the widely studied cytochrome *b* gene ranging from 10.6% to 12.8 % between *Salamandra atra* and S. s*alamandra*. These values are close to those found by García-París *et al.* (2003) between *S. salamandra* and *S. algira* (10.4%) and by Caccone *et al.* (1997) between *Euproctus platycephalus* and *E. montanus* (11.7 %). Several other case studies of congeneric species of amphibians presenting comparable degrees of divergence at cytochrome *b* sequences have been reviewed by Johns & Avise (1998). These authors evidenced how in the majority of pairwise congeneric species comparisons, K2P divergence ranges from 10 to 20 % in amphibians. But to our knowledge, no case study has been described to date showing two conspecific lineages of salamander diverging at cytochrome *b* gene sequences to as great an extent as those we found within *Salamandrina terdigitata*.

Following the above arguments, the two well-differentiated evolutionary lineages found within the spectacled salamander might represent two distinct taxa, for which we propose the rank of species. As will be discussed below, we suggest the name *Salamandrina perspicillata* for the northern species, while *S. terdigitata* should be retained for the southern one.

A substantial genetic homogeneity was found within both species, notwithstanding the fact that within *S. terdigitata* the populations from Calabria are distinctive according to allozymes. For several populations of *Salamandrina*, levels of genetic diversity resemble the average values reported by Nevo & Beiles (1991) for representatives of the family Salamandridae (H_E = 0.058; P = 24.0%). However, the samples of *S. terdigitata* from Amalfi and Serino showed practically no genetic diversity. This could be a result of either human-driven depletion such as that caused by habitat destruction, population isolation, etc. (e.g. Haig, 1998), or the marginal position of these populations within the species range (Ledig, 1986), or

from a combination of both factors. At this time we are unable to distinguish between these possible causes, even if the poor conservation status and fragmentation of the habitats in the Italian peninsula are likely to have played some role.

2.5 NOMENCLATURE DESIGNATION

As mentioned by Lanza (1988), the spectacled salamander was originally described by Lacépède (1788) with the name *Salamandra ter-digitata* on the basis of a single specimen collected by M. le Comte de Milly from Vesuvius. Fifteen years later, the species was also described (under the name *Salamandra tridactyla*) by Daudin (1803), who based his description on a specimen collected by De Nesle in the same locality. Later, another description was published by Savi (1821), who described the new species *Salamandra perspicillata* from cool and shady sites of Tuscan Apennine and particularly Mugello. He believed that he had found a new salamander species, because Lacépède's salamander was described as having four toes on the hind feet, but only three toes on the front feet. Later studies indicated that the two salamanders belonged to the same species, the original description by Lacépède being based on a poorly preserved specimen. Following the principle of priority of the International Code of Zoological Nomenclature, the species was given the name chosen by Lacépède, changed into *terdigitata* by removing the hyphen. Finally, Fitzinger (1826) proposed that the species belonged to a distinct monotypic genus, which he named *Salamandrina*. Other names have subsequently been proposed for the species, but they were all later synonymised (e.g. *Salamandra imperati* Costa, 1828; *Salamandra savi* Cuvier, 1829).

According to the principle of chronological priority and the geographic origin of our samples, we suggest that the name *Salamandrina perspicillata* (Savi, 1821) be used for the species from central Italy, while the name *Salamandrina terdigitata* (Lacépède, 1788) be retained for the southern species. However this new nomenclatural arrangement will need to be further discussed, depending on the identity of the type specimens being confirmed. The analyses of type specimens will

follow the completion of morphological analyses, now in progress, to identify potential morphological differences of diagnostic value between the two species. At present the two species can be diagnosed based on their genetic divergence, as assessed at both mtDNA sequence (Cytochrome-b and 12s rRNA genes) and diagnostic allozyme loci (*Mdhp1, Mdhp2, Gapdh, Aat1, Alat, PepB1, PepD2, Tpi, Pgm2*). Moreover, the geographic origin of specimens is also of diagnostic value, although the species assignment of populations from the central portion of the Volturno river drainage basin still needs to be assessed (see Figure 1).

2.6 CONCLUSION

The main outcome of this study has been the recognition of two distinct species within the Italian endemic *Salamandrina terdigitata*. Future efforts will be focused on two main fields: morphological variation, and genetic analyses of intermediate populations.

In many amphibians, previously undescribed morphological variation has often been assessed after species recognition in genetic studies (e.g. Nascetti *et al.*, 1988; Nascetti *et al.*, 1995). Since no studies to date have investigated morphological variation within the spectacled salamander, future studies will attempt to identify potential differences of diagnostic value between *S. terdigitata* and *S. perspicillata* at the level of chromatic, morphological and osteological characters. However, preliminary observations suggest that some chromatic differences do exist between the species. The ventral surface of the tail exhibits a bright red in *S. terdigitata*, whereas it looks reddish to brownish-orange in *S. perspicillata*. In addition, the demarcation between the dorsal and ventral coloration of the tail is sharper in *S. terdigitata* than in *S. perspicillata*.

With respect to genetic studies, further sampling will be focused on presumed or potential contact zones, such as mid-altitude areas of the Volturno river drainage basin (see Figure 1). The main objectives of the study of these populations will be: 1) to define the reciprocal distribution of the two species (i.e., if allopatric or parapatric); 2) to ascertain whether contact zone does exist and, in that

case, to delimitate it and to address what kind of genetic and ecological interactions are occurring between both species in this area.

Finally, the split of the former *S. terdigitata* into two distinct species with restricted geographical distributions, strongly suggests the necessity of a careful evaluation of their conservation status, as well as the importance that they be included in the main international lists of threatened species, such as the IUCN Red List.

2.7 TABLES AND FIGURES

Table 1. Geographic origin and sample size (n) of the 12 populations of *S. terdigitata* studied.

Sample code	Locality	Altitude (m a.s.l.)	Region	n	
				Allozymes	mtDNA
1	Bagno di Romagna	460	Emilia-Romagna	20	3
2	Barbarano Romano	340	Latium	9	4
3	Tolfa	480	Latium	7	5
4	Percile	575	Latium	11	5
5	Bassiano	560	Latium	12	6
6	M.te San Biagio	140	Latium	10	4
7	Serino	630	Campania	18	4
8	Amalfi	15	Campania	12	5
9	S. Severino Lucano	880	Basilicata	14	5
10	Viggianello	560	Basilicata	8	4
11	Taverna	670	Calabria	18	8
12	Stilo	520	Calabria	10	9

Table 2. Enzymes studied in *S. terdigitata*, their commission number (EC), encoding loci, buffer systems and tissues used in electrophoresis (M = skeletal muscle, L = liver).

Enzyme	EC	Encoding loci	Buffer systems	Tissue
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3pdh*	5	M
Lactate dehydrogenase	1.1.1.27	*Ldh-1*	4	M
		Ldh-2	4	M
Malate dehydrogenase	1.1.1.37	*Mdh-1*	5	M
Malate dehydrogenase ($NADP^+$)	1.1.1.40	*Mdhp-1*	2,5	M, L
		Mdhp-2	2,5	M, L
Isocitrate dehydrogenase	1.1.1.42	*Icdh-1*	6	M
		Icdh-2	6	M
6-Phosphogluconate dehydrogenase	1.1.1.44	*6Pgdh*	5	M
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	1	M
Glyceraldehyde-3-phosfate dehydrogenase	1.2.1.12	*Gapdh*	6	M
Superoxide dismutase	1.15.1.1	*Sod-1*	3	M
Aspartate transaminase	2.6.1.1	*Aat-1*	5	L, M
		Aat-2	5	L, M
Alanine transaminase	2.6.1.2	*Alat*	2, 7	L
Creatine kinase	2.7.3.2	*Ck*	2	L
Adenylate kinase	2.7.4.3	*Adk*	2	L
L-LeucylGlycylGlycine Peptidase	3.4.13	*Pep-B1*	7	M, L
		Pep-B2	7	M, L
L-Phenylalanyl-L-proline Peptidase	3.4.13.9	*Pep-D1*	2, 7	M, L

Table 2. (continue)

		Pep-D2	2, 7	M, L
Carbonic anhydrase	4.2.1.1	*Ca-2*	3	M
Triose-phosphate isomerase	5.3.1.1	*Tpi*	2	L
Mannose phosphate isomerase	5.3.1.8	*Mpi*	3	M
Glucose phosphate isomerase	5.3.1.9	*Gpi*	3	M
Phosphoglucomutase	5.4.2.2	*Pgm-1*	4	M
		Pgm-2	4	M
		Pgm-3	4	M
		Pgm-4	4	M

Buffer systems: 1) Discontinous Tris/Citrate pH 8.7 (Poulik, 1957); 2) Continous Tris/Citrate pH 8.0 (Selander *et al.*, 1971); 3) Tris/Versene/Borate pH 8.0 (Brewer & Sing, 1970); 4) Tris/Maleate pH 7.4 (Brewer & Sing, 1970); 5) Phosfate-Cytrate pH 6.3 (Harris, 1966); 6) Histidine/Citrate pH 7 (Cheliak & Pitel, 1984); 7) Lithium-borate pH 8.3 (Soltis *et al.*, 1983).

Table 3. Allele frequencies and estimates of genetic variability at 16 polymorphic loci for the twelve populations of *Salamandrina terdigitata* sampled. Populations are numbered as in Table 1.

Population	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Locus												
Ldh1												
100	0.79	0.83	0.90	0.95	0.93	1.00	0.17	-	0.23	0.06	0.97	0.80
110	0.21	0.17	0.10	0.05	0.07	-	-	-	-	-	-	-
112	-	-	-	-	-	-	0.83	1.00	0.77	0.94	0.03	0.20
Mdhp1												
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
102	0.17	-	-	-	-	-	-	-	-	-	-	-
104	0.83	1.00	1.00	1.00	0.36	0.25	-	-	-	-	-	-
108	-	-	-	-	0.64	0.75	-	-	-	-	-	-
Mdhp2												
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
112	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
Icdh2												
88	1.00	1.00	1.00	1.00	1.00	1.00	-	-	0.27	0.42	0.50	0.44
100	-	-	-	-	-	-	1.00	1.00	0.73	0.58	0.50	0.56
Gapdh												
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
110	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-

Table 3. (continue)

Aat1												
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
120	0.50	1.00	0.88	1.00	1.00	0.25	-	-	-	-	-	-
130	0.28	-	0.12	-	-	0.75	-	-	-	-	-	-
140	0.22	-	-	-	-	-	-	-	-	-	-	-
Alat												
70	0.10	-	-	-	-	-	-	-	-	-	-	-
80	0.90	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
PepB1												
95	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
PepD2												
100	-	-	-	-	-	-	1.00	1.00	0.94	0.88	1.00	1.00
105	0.17	-	-	0.06	-	-	-	-	-	-	-	-
110	0.83	1.00	0.88	0.56	0.50	0.85	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-	0.06	0.12	-	-
120	-	-	0.12	0.38	0.50	0.15	-	-	-	-	-	-
Ca2												
90	-	-	-	-	-	-	-	-	-	0.19	0.25	0.40
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.81	0.75	0.60
Tpi												
100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
105	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
Mpi												
95	0.70	0.69	0.60	0.70	0.83	0.85	-	-	-	-	-	-
100	0.30	0.31	0.40	0.30	0.17	0.15	1.00	1.00	1.00	1.00	1.00	0.95
106	-	-	-	-	-	-	-	-	-	-	-	0.05

Table 3. (continue)

	Gpi											
	95	0.15	0.44	0.30	0.23	0.29	0.25	-	-	-	-	-	-
	100	0.85	0.56	0.70	0.77	0.71	0.75	1.00	1.00	1.00	1.00	1.00	1.00
	Pgm2												
	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	103	-	-	-	-	0.07	0.30	-	-	-	-	-	-
	106	1.00	1.00	1.00	1.00	0.93	0.70	-	-	-	-	-	-
	Pgm3												
	90	-	-	-	-	-	-	-	-	-	-	0.03	-
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00
	108	-	-	-	-	-	-	-	-	-	-	0.03	-
	Pgm4												
	90	-	-	-	-	-	-	-	-	-	-	0.04	-
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.92	1.00	0.89	0.95
	106	-	-	-	-	-	-	-	-	0.08	-	0.07	0.05
P_{95}		24.1	10.3	17.2	10.3	20.7	20.7	3.4	0.0	13.8	13.8	13.8	17.2
A		1.3	1.1	1.2	1.2	1.2	1.2	1.0	1.0	1.1	1.1	1.2	1.2
		(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.0)	(0.0)	(0.1)	(0.1)	(0.1)	(0.1)
H_E		0.08	0.04	0.06	0.05	0.07	0.08	0.01	0.00	0.04	0.04	0.04	0.06
		(0.03)	(0.02)	(0.03)	(0.03)	(0.03)	(0.03)	(0.01)	(0.00)	(0.02)	(0.02)	(0.02)	(0.03)
H_O		0.09	0.04	0.06	0.03	0.07	0.07	0.01	0.00	0.04	0.03	0.03	0.06
		(0.03)	(0.02)	(0.03)	(0.02)	(0.03)	(0.03)	(0.01)	(0.00)	(0.02)	(0.02)	(0.01)	(0.03)

P_{95} = percentage of polymorphic loci with the most common allele not exceeding 95%. A = mean number of alleles per locus. H_E = average expected heterozygosity assuming Hardy-Weinberg equilibrium. H_O = average observed heterozygosity. Standard errors in parentheses.

Table 4. Pairwise values of standard Nei's (1972) genetic distances among the 12 populations of *Salamandrina terdigitata* sampled. Populations are numbered as in Table 1.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1	-											
2	0.01	-										
3	0.01	0.00	-									
4	0.01	0.01	0.00	-								
5	0.03	0.03	0.02	0.02	-							
6	0.03	0.05	0.04	0.05	0.03	-						
7	0.47	0.51	0.49	0.49	0.49	0.49	-					
8	0.48	0.52	0.50	0.51	0.51	0.50	0.00	-				
9	0.45	0.48	0.46	0.46	0.47	0.46	0.00	0.01	-			
10	0.45	0.48	0.47	0.47	0.47	0.47	0.01	0.01	0.00	-		
11	0.41	0.45	0.43	0.43	0.43	0.42	0.04	0.05	0.02	0.03	-	
12	0.42	0.46	0.44	0.44	0.44	0.43	0.03	0.04	0.02	0.02	0.00	-

Table 5. AMOVA results for *S. terdigitata* data as obtained using ARLEQUIN 2.000 (Schneider *et al.*. 1999). Groups were defined as the two main clusters identified by the UPGMA cluster analysis (Figure 2).

Source of variation	Percentage of variation	Fixation indices	*P*-values
Among groups	91.38	F_{CT} = 0.914	< 0.01
Among populations within groups	1.47	F_{SC} = 0.171	< 0.01
Within populations	7.14	F_{ST} = 0.929	< 0.01

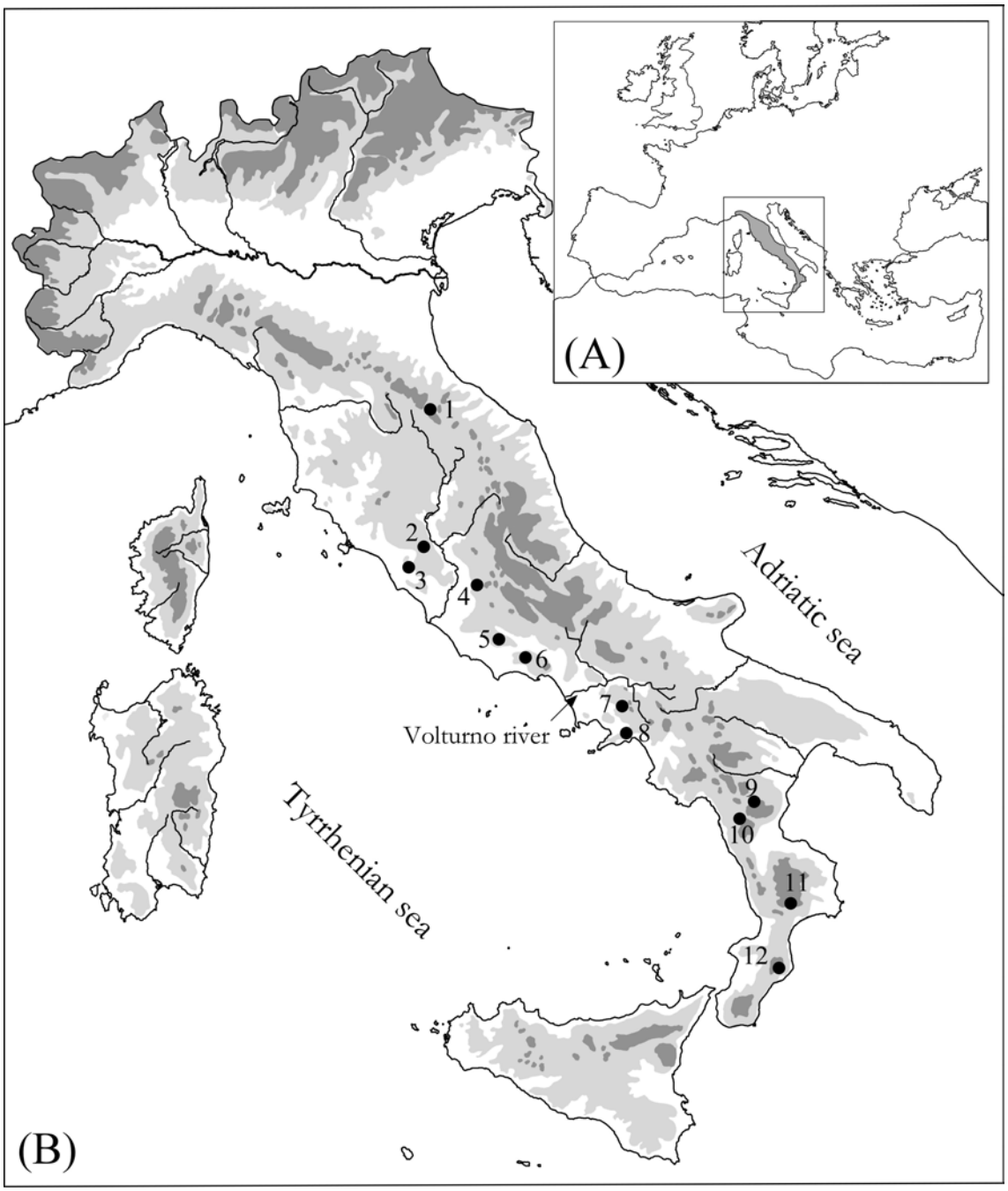

Figure 1. Species range (A) and geographic location of the 12 populations sampled of *S. terdigitata* (B). Localities are numbered as in Table1.

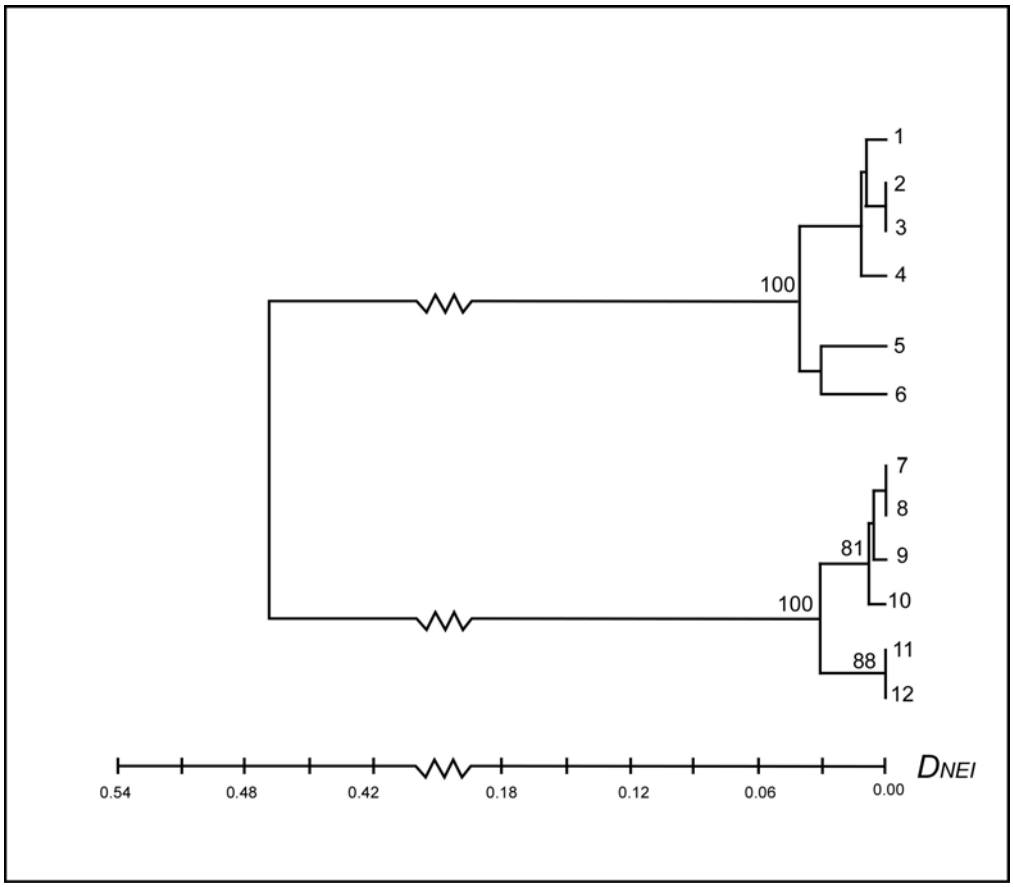

Figure 2. UPGMA tree showing genetic relationships among the 12 populations of *S. terdigitata*, based on Nei's (1972) standard genetic distance values. Bootstrap values > 70% over 1000 pseudoreplicates are indicated. Populations are numbered as in Table 1.

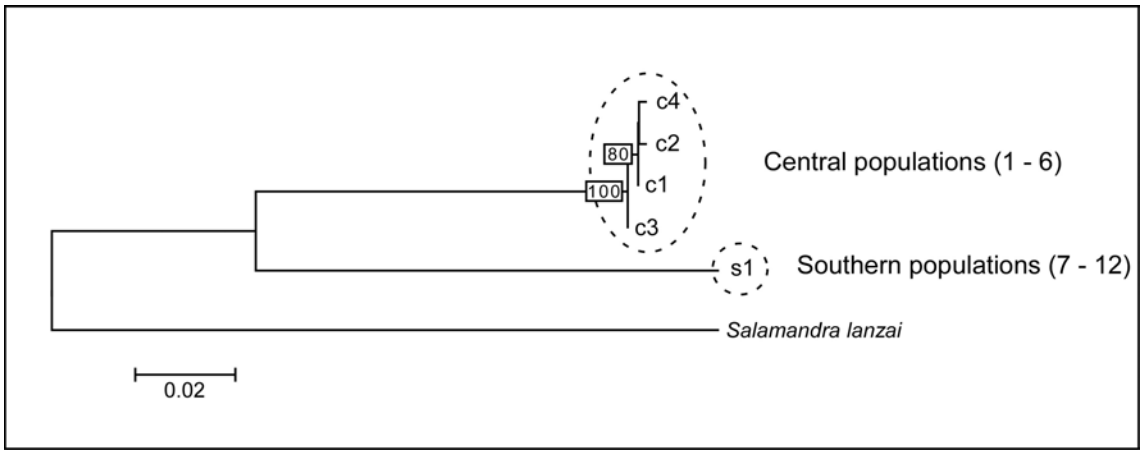

Figure 3. Neighbour Joining tree based on Kimura-2-parameter divergence values (K2P) for cytochrome *b* haplotypes (629bp) observed within *S. terdigitata*. Bootstrap values over 1000 pseudoreplicates are shown at the nodes when exceeding 70% .

3. THE SPECTACLED SALAMANDERS, *Salamandrina terdigitata* LACÉPÈDE, 1788 AND *S. perspicillata* SAVI, 1821: 1) GENETIC DIFFERENTIATION AND EVOLUTIONARY HISTORY.

GIUSEPPE NASCETTI, FRANCESCA ZANGARI & DANIELE CANESTRELLI.

ABSTRACT

Recent genetic investigations revealed the existence of two distinct species within the former *Salamandrina terdigitata*, once considered the only representative of its Italian endemic genus. The pattern of genetic differentiation between S*alamandrina terdigitata* and *S. perspicillata*, as previously assessed with both allozyme and mitochondrial markers, was further analyzed and compared with that observed among other congeneric species of European salamanders. Partial sequences of cytochrome *b* and 12S rRNA mtDNA genes were therefore obtained from four European species of the genus *Salamandra*. Genetic differentiation was particularly high within *Salamandrina* at mitochondrial level, with values of K2P divergence (17 % and 6 % for cytochrome *b* and 12s respectively) exceeding those of many other species of related salamander. The presence of a *Salamandrina* Miocenic fossil record from Sardinia suggests that the common ancestor of *S. terdigitata* and *S. perspicillata* could have been a Corsica-Sardinian element. The distribution and the depth of genetic divergence between the two species could be the result of a vicariance event, through the separation of the Calabro-Peloritan massif from Sardinia (7.8-8.6 Myr ago), followed by a subsequent dispersal from Corsica-Sardinia plate toward the continent during late Messinian (about 5-6 Myr ago), or through one of the Plio-Pleistocenic land-bridges between the Corsica-Sardinia plate and the Tuscan Archipelago.

KEYWORDS: spectacled salamanders, *Salamandrina terdigitata*, *Salamandrina perspicillata*, genetic divergence, biogeography.

3.1 INTRODUCTION

The Italian endemic *Salamandrina* FITZINGER,1826 (Urodela: Salamandridae) is the only European endemic terrestrial vertebrate genus (Lanza, 1988). According to the morphological and molecular analysis of Titus & Larson (1995), it is considered an ancient lineage that separated from the other newt lineages around the time of the split between the remaining newts and the true salamanders. Fossil records have revealed that its distribution was once wider, comprising at least Sardinia and Greece (e.g. Vanni & Nistri, 1997).

Salamandrina terdigitata LACÉPÈDE, 1788 has long been considered the only living species of the genus. However, recent genetic studies based on both nuclear and mitochondrial markers (Canestrelli *et al.*, in press), revealed the existence of two well differentiated species within the spectacled salamander. Consequently, populations spanning from Liguria to central Campania have been recognized under the name *Salamandrina perspicillata* SAVI, 1821, whereas the name *Salamandrina terdigitata* LACÉPÈDE, 1788 was retained for populations from central Campania to the tip of Calabria. The Volturno river drainage basin (Figure 1) was suggested as the area of possible close contiguity between the two species, although their reciprocal distribution (i.e., if allopatric or parapatric) has not yet been clarified.

In this paper, the pattern of genetic divergence between *Salamandrina terdigitata* and *S. perspicillata* is further investigated and compared with that observed among other species of European salamanders. Moreover, hypotheses about the historical events which led to the observed pattern of divergence are discussed.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING

Two distinct sampling sessions were performed: spring-autumn 1990 and spring-autumn 2002. During the first sampling session, specimen were collected from twelve populations covering the whole range of the genus (Figure 1). Each

individual was carried to the laboratory, euthanasized with an excess of 3-aminobenzoic acid ethyl ester (MS222) and then samples of skeletal muscle and liver were removed and stored at –80 °C. These specimens were used to adjust experimental protocols and to score liver-active enzymes. The second sampling session was carried out in the same collection sites, but this time each specimen was anesthetized in the field with MS222 following suggestions given by Heyer *et al.* (1994) and tail-clipped (2-3 cm) before being released in the same place. Tissue samples were carried to the laboratory in liquid nitrogen containers and stored at -80 °C to await further analysis. Geographic origin of samples studied and sample size are given in Table 1.

3.2.2 ALLOZYME ANALYSIS

Standard horizontal starch gel electrophoresis was used to study genetic variation at level of 20 enzymes encoded by 29 presumptive loci. The following loci were studied: *G3pdh, Ldh-1, Ldh-2, Mdh-1, Mdhp-1, Mdhp-2, Icdh-1, Icdh-2, 6Pgdh, G6pdh, Gapdh, Sod-1, Aat-1, Aat-2, Alat, Ck, Adk, Pep-1, PepB-2, PepD-1, PepD-2, Ca-2, Tpi, Mpi, Gpi, Pgm-1, Pgm-2, Pgm-3, Pgm-4*. Details of the electrophoretic techniques implemented can be found in Canestrelli *et al.* (in press).

Genetic distances among population were estimated using Nei's (1972) standard genetic distance (D_{NEI}) and Rogers (1972) distance (D_R), using the software BIOSYS-2 (Swofford & Selander, 1999). D_{NEI} was then used to build an UPGMA phenogram, whose reliability was assessed with 1000 bootstrap replicates over loci.

3.2.3 MTDNA ANALYSIS

As described in Canestrelli *et al.* (in press), genomic DNA was extracted following standard phenol:chloroform protocol (Sambrook *et al.*, 1989). Partial sequences of the mitochondrial cytochrome *b* gene (629bp) and 5'-end of the 12S rRNA (436 to 444 bp) were obtained by means of PCR-amplification using primers' pairs MVZ15-MVZ16 and 12SZL-12SFH respectively (Moritz *et al.*, 1992; Goebel *et al.*, 1999), followed by double-sequencing on a ABI3730 XL automatic DNA sequencer. In this study, the 3'-end of the 12S rRNA fragment was also sequenced in order to obtain the almost complete sequence of this mtDNA region for all the

previously sequenced specimens. With this aim, a PCR-amplification was carried out using the primers' pair 12SAL-tRNAvalH (Palumbi *et al.*, 1991; Goebel *et al.*, 1999) and PCR protocol as for the 5'-end fragment. Since the two 12S rRNA fragments were partially overlapping, they were here joined and analyzed as a single sequence (836-844 bp).

For comparative purposes, partial sequences of the same two mitochondrial genes were also obtained from alcohol preserved specimens of the following species (Nascetti legit; two specimens each): *Salamandra salamandra* from Volpaia (Italy), *S. corsica* from Vizzavona (Corsica), *S. atra* from Asiago (Italy) and *S. lanzai* from Pian del Re (Italy). Sequence for *S. luschani* was obtained from GenBank (Accession Numbers: AF154053).

The sequences were aligned using CLUSTALX 1.81 (Thompson *et al.*, 1997). All the haplotypes found were deposited in Genbank (Accession Numbers: AY695901- AY695907, AY928613-AY928621).

Number of substitutions and genetic distances between haplotypes according to Kimura-2-parameters model (K2P; Kimura, 1980) were assessed using the software MEGA 2.1 (Kumar *et al.*, 2001). This software was also used to implement the Tajima's relative rate test (1993), under the null hypothesis of constant substitution rate among sequences. *Ambystoma mexicanum* was used as outgroup (Accession Number: NC005797).

3.3 RESULTS

3.3.1 ALLOZYMES

As shown in Table 2, genetic distances between the two species ranged from D_{NEI}=0.41 to D_{NEI}=0.52 and from D_R=0.37 to D_R=0.42, with average values of D_{NEI}=0.47 (0.03 SD) and D_R=0.39 (0.01 SD). Within *S. perspicillata*, D_{NEI} varies from 0.00 to 0.05 (mean: 0.02 [0.02 SD]), whereas D_R from 0.02 to D_R=0.09 (mean: 0.05 [0.02 SD]). Similar values were also found within *S. terdigitata*, with D_{NEI}

ranging from 0.00 to 0.05 (mean: 0.02 [0.01 SD]) and D_R from 0.01 and 0.06 (mean: 0.04 [0.02 SD]).

This pattern is reflected in the UPGMA analysis (Figure 2), where populations of each species clustered together achieving 100% of bootstrap support.

3.3.2 MTDNA

For the spectacled salamanders, partial sequences of 629 bp and 836-844 bp were obtained for cytochrome *b* and 12S rRNA respectively.

At the cytochrome *b* fragment, all *S. terdigitata* specimens shared a single haplotype (s1), that was never found within *S. perspicillata* samples. In these latter samples, four haplotypes (c1-c4) were found differing at most in two nucleotide substitutions. However, all but three specimens shared the haplotype c1 (haplotypes c3 and c4 were both observed only once in the sample 4, whereas the haplotype c2 was found in one specimen from sample 1). Within *Salamandrina*, cytochrome *b* haplotypes were defined by 95 variable positions (79 at the third position, 11 at the first and 5 at the second, with a comprehensive number of 9 aminoacid replacements), and lead to an average genetic distance between the two species of K2P = 0.17 (0.02 SD).

Not surprisingly, genetic divergence between the two species was less pronounced at the 12S rDNA gene. In fact, only two distinct haplotypes were found, alternatively fixed within the two species, differing at level of 45 nucleotide substitution and 5 indels (4 of 1 bp and one of 6 bp in length), with a genetic distance of K2P = 0.06.

Pairwise genetic divergence among the five European species of the genus *Salamandra*, based on partial sequences of cytochrome *b* (486 bp) and 12S rRNA (824-837 bp) is shown in Table 3. Within this group of species, ranges of K2P divergence were 0.06-0.22 and 0.03-0.09 for cytochrome *b* and 12S respectively, with the most differentiated species being *S. luschani*. This latter species showed an average K2P divergence of 0.20 (0.02 SE) and 0.08 (0.01 SE) at cytochrome *b* and 12S respectively, compared with the other *Salamandra* species.

The null hypothesis of constant substitution rate, as assessed by the Tajima's relative rate test (1993), couldn't be rejected for any of the 42 tests performed (all $P > 0.01$).

Assuming 12 Myr of divergence between *S. luschani* and the other *Salamandra* species (according to Weisrock *et al.*, 2001), the divergence rate for the mtDNA genes studied here could be estimated at 1.67% and 0.67 % per million years for cytochrome *b* and 12S respectively.

3.4 DISCUSSION

The recent finding of two well differentiated species within the former *Salamandrina terdigitata* was a really unexpected result (Canestrelli *et al.*, in press), especially when considering its limited geographic range (restricted to the Apennine chain [e.g. Lanza, 1983a]) as well as the apparent lack of chromatic or morphological variation (with the exception of few chromatic variant described at local level [reviewed by Lanza & Canestrelli, 2002]).

Levels of differentiation found at allozymes between *Salamandrina terdigitata* and *S. perspicillata* (D_{NEI} = 0.47, D_R=0.39), resemble that found between several other congeneric species of salamander. For instance, among three *Hydromantes* species from mainland Italy, Nascetti *et al.* (1996) found values of D_{NEI} ranging from 0.33 to 0.40, whereas among three *Hydromantes* species from eastern Sardinia the same authors (see also Lanza *et al.*, 1995) reported values of D_{NEI} ranging from 0.47 to 0.48. Within the genus *Triturus* (Macgregor *et al.*, 1990), similar values were found between the species pair *T. carnifex* - *T. cristatus* (D_{NEI} =0.38), *T. carnifex* – *T. pygmaeus* (D_{NEI} =0.58), *T. vulgaris meridionalis* – *T. pygmaeus* (D_{NEI} =0.52) and between *T. pygmaeus* and *T. marmoratus* (D_{NEI} =0.19) which have only recently been recognized as distinct species (Garcia-Paris *et al.*, 2001).

Genetic differentiation between *Salamandrina terdigitata* and *S. perspicillata* resulted particularly high at mitochondrial level. In fact, values of K2P divergence observed among them (17 % and 6 % for cytochrome *b* and 12s respectively)

exceed those achieved by many other species of related salamander. For instance, in the majority of pairwise comparisons among European species of the genus *Salamandra* (see Table 3), we found values of K2P lower (in same case the half) than that found within *Salamandrina*. For comparative purpose, we have also recalculated values of K2P for sequences of cytochrome *b* gene from *Pleurodeles* and related genera, from recently published data (Veith *et al.*, 2004). As shown in Table 4, K2P genetic divergence between *Salamandrina terdigitata* and *S. perspicillata* (17%) results much higher than that found within the genus *Pleurodeles* and, astonishingly, equals or resembles that found between distinct genera (but "naïve" speculations about taxonomic implications of this observation should be avoided).

The lack of fossil records within the present range of the genus *Salamandrina* and the lack of a clear geographic barrier to dispersal in the putative area of close contiguity between *S. terdigitata* and *S. perspicillata*, permit us neither to unambiguously indicate an historical event nor to directly calibrate molecular clocks, in an attempt to explain the observed pattern and depth of genetic divergence between the two species. Therefore, both tempo and mode of divergence between them have to be estimated indirectly.

By applying to allozymes data a divergence rate of to 0.07 D_{NEI} / Myr, estimated for plethodontid salamanders (Maxson & Maxson, 1979), the time of divergence between *S. terdigitata* and *S. perspicillata* was estimated at 6.71 ($\pm$0.43) Myr ago. For mitochondrial sequences, the divergence rates of 1.67% and 0.67% per million year for cytochrome *b* and 12S respectively, lead to a split-time estimate of 8.96-10.18 Myr ago. Although estimates based on allozymes and mtDNA data don't overlap, and regardless of the vagaries of molecular clocks (e.g. Ayala, 1997), the depth of genetic differentiation between *S. terdigitata* and *S. perspicillata* suggests that their divergence began in the upper Miocene (11-5 Myr ago).

The presence of a *Salamandrina* Miocenic fossil record from Sardinia suggests that the common ancestor of *S. terdigitata* and *S. perspicillata* could have been a Corsica-Sardinian element. Following this argument, and considering the depth of genetic differentiation found between the two species, the most probable paleogeographic event leading to their divergence could have been the separation of

the Calabro-Peloritan massif from Sardinia (7.8- 8.6 Myr ago, according to Duermeijer *et al.*, 1998). One lineage (the present *S. terdigitata*) would have reached southern Italy carried by the eastward migration of the Calabro-Peloritan massif, whereas the presence of the second species in Italy (the present *S. perspicillata*) would be explained by a dispersal event from the Corsica-Sardinian plate to the continent. Environmental conditions favourable to this dispersal could have occurred during late Messinian (about 5-6 MY), when freshwater habitats were present in the Mediterranean basin (e.g. Krijgsman *et al.*, 1999; Rouchy *et al.*, 2001; Rouchy *et al.*, 2003; Steinfartz *et al.*, 2001). Alternatively, peninsular Italy could have been reached through by one of the Plio-Pleistocenic land-bridges between the Corsica-Sardinian plate and the Tuscan Archipelago, as has also been suggested for other faunal dispersal (e.g. Lanza, 1983b).

3.5 TABLES AND FIGURES

Table 1. Geographic origin and sample size (n) of the 12 populations studied of the genus *Salamandrina*.

Species	Sample code	Locality (region)	n	
			Allozymes	mtDNA
S. perspicillata	1	Bagno di Romagna (Emilia-Romagna)	20	3
	2	Barbarano Romano (Latium)	9	4
	3	Tolfa (Latium)	7	5
	4	Percile (Latium)	11	5
	5	Bassiano (Latium)	12	6
	6	M.te San Biagio (Latium)	10	4
S. terdigitata	7	Serino (Campania)	18	4
	8	Amalfi (Campania)	12	5
	9	S. Severino Lucano (Basilicata)	14	5
	10	Viggianello (Basilicata)	8	4
	11	Taverna (Calabria)	18	8
	12	Stilo (Calabria)	10	9

Table 2 Values of Nei (1972) standard genetic distance (below the diagonal) and Rogers (1972) distance (above the diagonal) between the twelve samples studied of genus *Salamandrina*. Samples are numbered as in Table 1.

Species	**Sample**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
S. perspicillata	**1**	-	0.04	0.04	0.04	0.07	0.07	0.39	0.39	0.38	0.38	0.37	0.37
	2	0.01	-	0.02	0.03	0.06	0.09	0.41	0.42	0.40	0.40	0.39	0.39
	3	0.01	0.00	-	0.02	0.05	0.07	0.40	0.41	0.39	0.39	0.38	0.37
	4	0.01	0.01	0.00	-	0.04	0.08	0.40	0.41	0.39	0.39	0.38	0.37
	5	0.03	0.03	0.02	0.02	-	0.05	0.40	0.41	0.39	0.39	0.38	0.37
	6	0.03	0.05	0.04	0.05	0.03	-	0.40	0.40	0.39	0.39	0.38	0.37
S. terdigitata	**7**	0.47	0.51	0.49	0.49	0.49	0.49	-	0.01	0.02	0.03	0.05	0.06
	8	0.48	0.52	0.50	0.51	0.51	0.50	0.00	-	0.02	0.03	0.06	0.06
	9	0.45	0.48	0.46	0.46	0.47	0.46	0.00	0.01	-	0.02	0.04	0.05
	10	0.45	0.48	0.47	0.47	0.47	0.47	0.01	0.01	0.00	-	0.04	0.05
	11	0.42	0.46	0.44	0.44	0.44	0.43	0.03	0.04	0.02	0.02	-	0.02
	12	0.41	0.45	0.43	0.43	0.43	0.42	0.04	0.05	0.02	0.03	0.00	-

Table 3 Values of pairwise K2P genetic distances for the European species of the genera *Salamandra* and *Salamandrina*, as estimated at level of the cytochrome *b* (486 bp; below the diagonal) and 12S rRNA (824-837 bp; above the diagonal) gene fragments. Standard errors are given in parentheses.

		1	**2**	**3**	**4**	**5**	**6**	**7**
1	*Salamandra atra*	-	0.03	0.04	0.04	0.07	0.16	0.15
			(0.01)	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)
2	*S. lanzai*	0.08	-	0.03	0.05	0.08	0.16	0.14
		(0.01)		(0.01)	(0.01)	(0.01)	(0.02)	(0.01)
3	*S. corsica*	0.06	0.07	-	0.04	0.09	0.16	0.15
		(0.01)	(0.01)		(0.01)	(0.01)	(0.02)	(0.02)
4	*S. salamandra*	0.10	0.08	0.09	-	0.09	0.16	0.15
		(0.02)	(0.01)	(0.01)		(0.01)	(0.02)	(0.01)
5	*S. luschani*	0.19	0.21	0.19	0.22	-	0.16	0.17
		(0.02)	(0.02)	(0.02)	(0.03)		(0.02)	(0.02)
6	*Salamandrina terdigitata*	0.27	0.27	0.28	0.28	0.33	-	0.06
		(0.03)	(0.03)	(0.03)	(0.03)	(0.03)		(0.01)
7	*Salamandrina perspicillata*	0.24	0.26	0.23	0.25	0.28	0.18	-
		(0.03)	(0.03)	(0.02)	(0.03)	(0.03)	(0.02)	

Table 4 Values of pairwise K2P genetic distance among cytochrome *b* gene sequences from the main lineages of the genus *Pleurodeles* and related genera. Modified from Veith *et al.* (2004). GenBank accession numbers are given in parentheses.

	Species	1	2	3	4	5	6	7	8	9
1	*Pleurodeles poireti* (AY336647)	-								
2	*Pleurodeles poireti* (AY336643)	0.01	-							
3	*Pleurodeles poireti* (AY3366419	0.08	0.08	-						
4	*Pleurodeles waltl* (AY336657)	0.09	0.10	0.10	-					
5	*Pleurodeles waltl* (AY336653)	0.10	0.11	0.09	0.04	-				
6	*Tylototriton verrucosus* (AY336660)	0.17	0.17	0.17	0.18	0.19	-			
7	*Salamandra salamandra* (AY336658)	0.17	0.17	0.17	0.19	0.18	0.18	-		
8	*Neurergus crocatus* (AY336661)	0.20	0.20	0.17	0.20	0.19	0.20	0.16	-	
9	*Triturus vittatus* (AY336659)	0.21	0.21	0.21	0.23	0.21	0.19	0.21	0.17	-

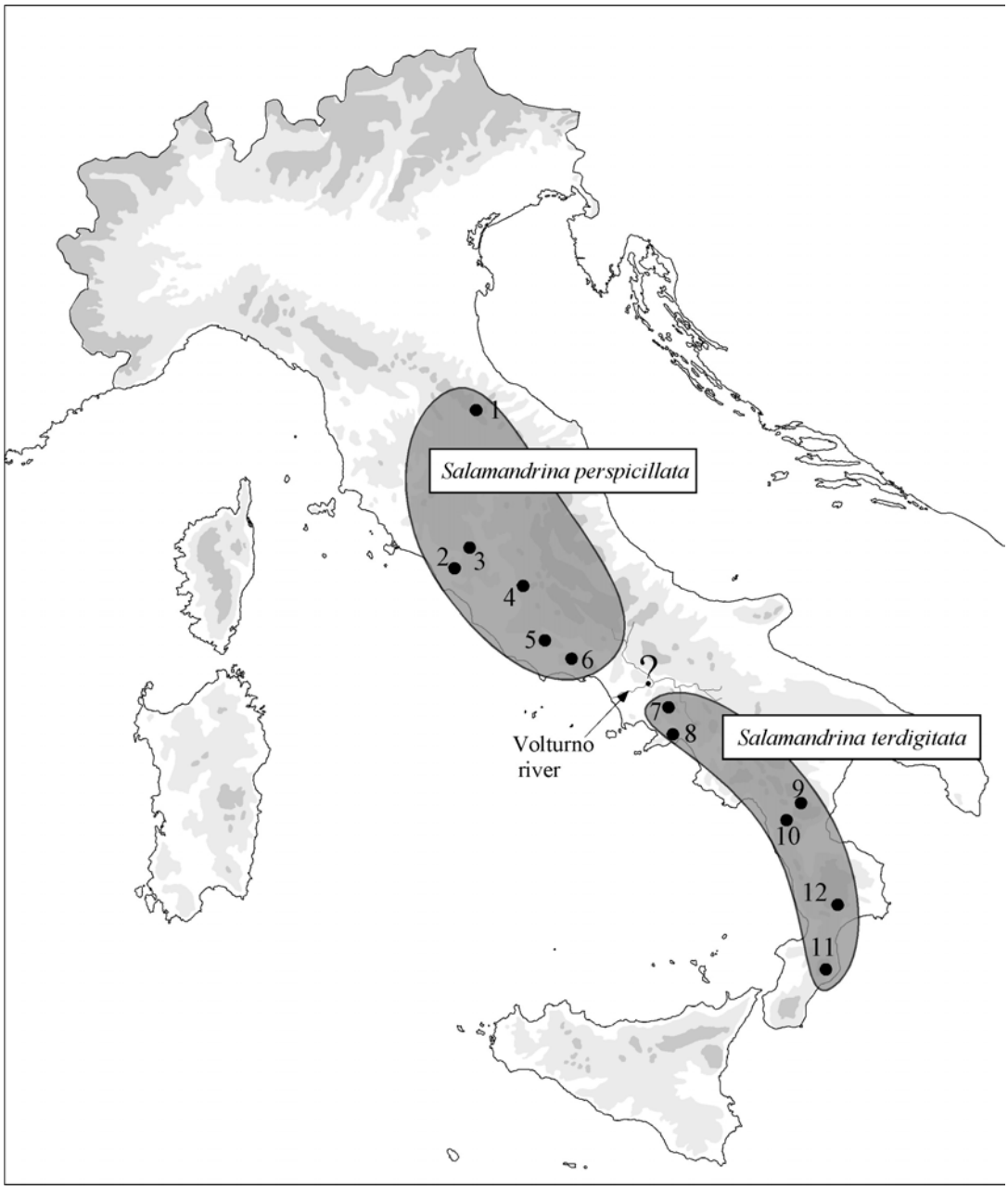

Figure 1. Geographic location of the 12 populations sampled of the genus *Salamandrina*. Question mark indicates the geographic area of probable close contiguity between *S. terdigitata* and *S. perspicillata*. Locality are numbered as in Table1.

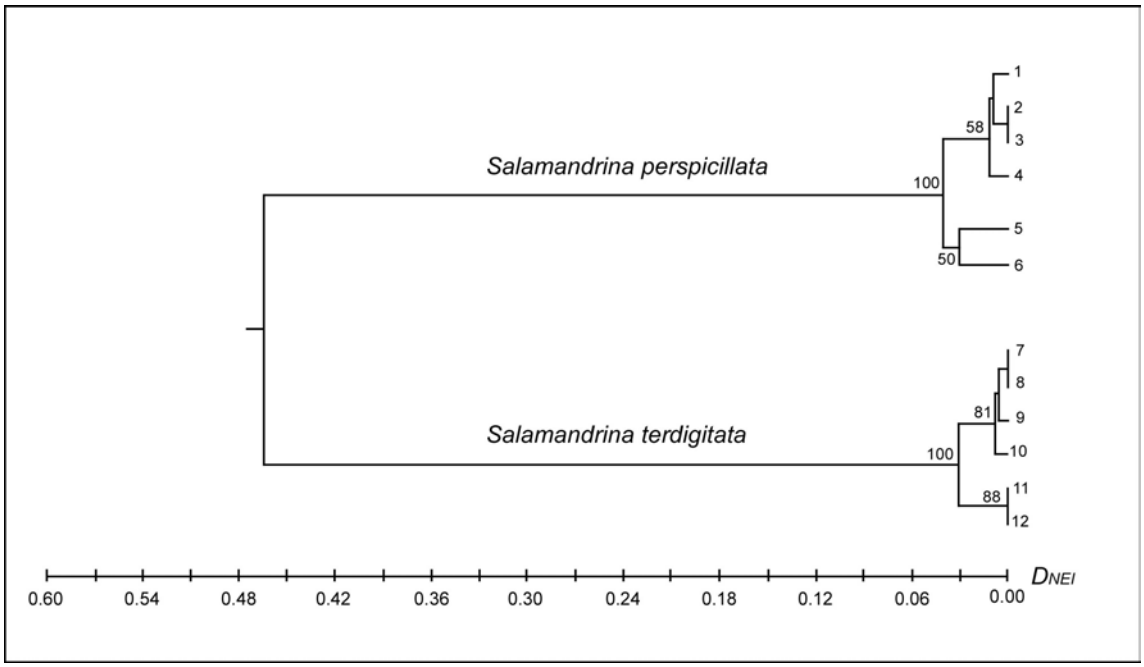

Figure 2. UPGMA phenogram for the twelve populations studied of the genus *Salamandrina*. Based on the matrix of standard Nei's (1972) genetic distances. Bootstrap support > 50% after 1000 replicates are shown at the nodes. Samples are numbered as in Table 1.

4. GENETIC DIFFERENTIATION AND HISTORY OF POPULATIONS OF THE ITALIAN TREEFROG AS REVEALED BY THE DISCREPANCY BETWEEN MITOCHONDRIAL AND NUCLEAR GENES.

DANIELE CANESTRELLI, ANDREA VERARDI & GIUSEPPE NASCETTI

ABSTRACT

The genetic differentiation among 33 populations of the Italian treefrog was investigated using both biparentally (23 allozyme loci) and maternally (partial mitochondrial cytochrome *b* gene) inherited genes. Two main population groups were evidenced by both markers, located north and south of thenorthern Apennines. However, the pattern of differentiation between these two groups was much less pronounced at allozymes than at mtDNA, leading to gene flow estimates that were 25 times higher at nuclear than at mitochondrial level. Also, the mtDNA divergence between the two groups was particularly marked for two cospecific lineages of anuran amphibians (the *p*-distance being on average 9.04%), while their average genetic distance at allozymes was comparatively low (D_{NEI}=0.07). The most convincing explanation for the observed pattern of genetic variation have appeared to imply both the maintenance of ancestral polymorphisms and a male-biased gene flow, along with several historical and demographic events.

Keywords: allozymes; mitochondrial DNA; Italian treefrog; population structure; discrepant genetic markers.

4.1 INTRODUCTION

The introduction of genetic markers in the study of geographical patterns of variation between natural populations has had important consequences on the general perception of the natural history of several taxa. For instance, in amphibians which are generally conservative in their morphological evolution (Cherty *et al.*, 1978; Richards & Moore, 1996), often phylopatric and with relatively low dispersal abilities (Marsh & Trenham, 2000; Jehle & Arntzen, 2002), a huge amount of studies have given evidence of the existence of cryptic biodiversity and complex population structures that were previously unsuspected (e.g. Veith, 1996).

For the study of genetic differentiation among natural populations, a wide array of markers have now became available, from both nuclear and organelle genomes (Avise, 2000 and 2004; Zhang & Hewitt, 2003; Ballard & Whitlock, 2004). The relative importance of nuclear vs. mitochondrial markers for the study of population genetics and evolutionary processes has long been discussed (e.g. Moore, 1995; Hoelzer, 1997; Avise, 2000; Hare, 2001), even following evidence that distinct markers can give discrepant estimates of genetic differentiation among populations, leading to discrepant inferences regarding populations' structure and history (e.g. Palumbi & Baker, 1994; Piel & Nutt, 2000; Shaw, 2002; Johnson *et al.*, 2003). Some differences among mtDNA and nuclear markers are usually expected, because of their different effective size (typically four times lower in the former; Birky *et al.*, 1989), but several other causes can contribute or even play a prevalent role in enhancing these differences. Because of the lower effective size of mtDNA, historical events implying demographic size changes, such as population bottlenecks or founder effects, are expected to affect genetic diversity at this marker more strongly than at nuclear ones. This leads to mtDNA lineages becoming exclusive more quickly in diverging groups. Gender-biased factors also have been shown significantly to affect the magnitude of differentiation at different marker loci. For instance a male-biased sex ratio, implying a reduced female effective size, would enhance the effect of genetic drift on the (maternally inherited) mtDNA compared to the (biparentally inherited) autosomal genome (e.g. Arnaud-Haond *et al.*, 2003).

On the other hand, a gender-biased dispersal or phylopatry can produce different patterns of distribution of genetic variation at mitochondrial and nuclear genomes, because of differences in rates of gene flow (e.g. Palumbi & Baker, 1994; Goudet *et al.*, 2002). Finally, even selection can produce discrepancies among distinct kinds of markers. In fact, fixation of new allele variants through directional selection (selective sweeps) can lead to increased estimates of differentiation among the diverging lineages, both at the selected locus and at those loci that are tightly linked to it (Maynard Smith & Haigh, 1974). If acting on a mitochondrial gene, this leads to the fixation of a single mtDNA variant, implying that a stronger mitochondrial differentiation will be found compared to nuclear genes, whichever mtDNA gene fragment we study (Schlotterer, 2003; Ballard & Whitlock, 2004). In contrast, balancing selection, promoting the maintenance of polymorphism, lead to a less pronounced differentiation at the affected loci with respect to those evolving under selective neutrality (Broughton & Harrison, 2003).

In this paper we present a new case of striking discrepancy between nuclear (allozymes) and mitochondrial markers in depicting the pattern and depth of differentiation among populations of the Italian treefrog, and discuss the possible contribution of different evolutionary processes in generating the observed pattern.

The Italian treefrog *Hyla intermedia* is a small, cryptically coloured amphibian which breeds in ponds, pools, temporary waters and various other freshwater habitats (Lanza, 1983). It is a relatively thermophilic species, with the majority of populations (> 90%) being distributed below 500m a.s.l. (e.g. Mazzotti *et al.*, 1999). Because of the scarcity of morphological and chromatic differentiation, the populations of this species were long attributed to the European species *Hyla arborea* (Linnaeus, 1758), together with all other Palaearctic populations of treefrog. Some authors (e.g. Tscudi; cited in Schreiber, 1985) even believed the species to be cosmopolitan. Later studies based on genetic and bioacoustic markers revealed the existence, within the Paleartic region, of several distinct species within the former *Hyla arborea* (e.g. Paillette, 1967; Schneider, 1974; Kawamura *et al.*, 1977; Nascetti *et al.*, 1985). Based on an allozyme survey, Nascetti *et al.* (1995) attributed the treefrog populations from peninsular Italy and Sicily to the new species *Hyla italica*, which showed a genetic divergence (Nei, 1972) of $D_{NEI} = 0.47$ with respect to the other

European populations regarded as *Hyla arborea*. Subsequently, a synonimizing of *Hyla italica* (Nascetti *et al.*, 1995) with *Hyla intermedia* (Boulenger, 1882), and the consequent replacing of the latter name with the former, was proposed (Dubois, 1995). We don't regard a renaming as justified based on the arguments therein. Nevertheless, since this latter name is presently the most widely used and a decision has not yet been made by the International Commission on Zoological Nomenclature, in this paper we adopt the name *H. intermedia*, postponing a discussion about nomenclatural issues to a further paper (Nascetti *et al.*, in prep.).

The allozyme analysis of Nascetti *et al.* (1995), in which the species is described, also suggested the existence of some genetic differentiation among populations located in northern Italy with respect to those located in peninsular Italy and Sicily. In this paper we aim at assess the present geographical pattern of differentiation among populations of the Italian treefrog and to elucidate the evolutionary processes involved in generating this pattern. With this aim, we further investigate the genetic variation at allozymes through a more intensive sampling scheme, and analyse the geographic variation at mitochondrial level through PCR-RFLP and sequence analyses of a Cytochrome-b gene fragment.

4.2 MATERIALS AND METHODS

Altogether 497 specimens of *Hyla intermedia* were collected from 33 populations, covering the entire species range (Figure 1). The geographic origin of samples studied and sample size are given in Table 1. Tissue samples were obtained through toe-clipping procedure, following the suggestions of Donnelly *et al.* (1994). Each sampled individual was then released in the same collection place. Collected samples were carried to the laboratory in liquid nitrogen containers, then stored at – 80 °C until further analysis could be carried out. Samples from Verucchio, San Daniele and Langhirano were provided by the Department of Animal and Human Biology of Turin University and had been stored in 70% ethanol.

4.2.1 ALLOZYMES

Horizontal electrophoresis was carried out onto 10% starch gel. Enzyme systems analysed and their putative encoding loci are listed in Table 2 together with the electrophoretic techniques utilized. Isozymes were numbered in order of decreasing mobility, from the most anodal one. Alleles at each locus were designated by their mobility (in mm, standardized conditions) relative to the most common one (*100*) in a reference population (Corleone, Sicily).

Estimates of allele frequencies and population genetic variability (as mean observed heterozygosity, Nei's 1978 unbiased estimate of expected heterozygosity, percentage of polymorphic loci and mean number of alleles per locus) were computed with the software BIOSYS-2 (Swofford & Selander, 1999). Exact significance probabilities for Hardy-Weinberg equilibrium (HW) were assessed for each locus in each sample, then the Bonferroni correction for multiple tests was applied (Rice, 1989).

Genetic distances between populations were assessed by calculating Nei's (1972) standard genetic distances, which was then used to build an UPGMA phenogram. 1,000 bootstrap pseudoreplicates over loci were run to test the reliability of the UPGMA phenogram with the BOOTDIST option in BIOSYS-2. The consensus UPGMA was then obtained using the subroutines NEIGHBOR and CONSENSE in the software PHYLIP 3.5c (Felsenstein, 1993). Because UPGMA analysis forces populations into a dichotomic branching pattern, we also carried out a Principal Component Analysis (PCA) with the software PCAGEN 1.2 (Goudet, 1999), in order to detect potentially intergraded populations relative to the main lineages detected. The statistical significance of each axis was evaluated over 10,000 randomizations.

4.2.2 MTDNA

DNA was extracted from frozen or alcohol-preserved tissues following standard Sambrook *et al.* (1989) extraction protocol. A fragment of 332 bp of the mitochondrial gene encoding for the Cytochrome-b was amplified by means of the polymerase chain reaction (hereon, PCR) using primers MVZ15 (Moritz *et al.*, 1992) and H15149 (Kocher *et al.*, 1989). PCR cycling protocol was: 95°C for 5 min

followed by 39 cycles of 93°C for 1 min, 45°C for 45 s, 72°C for 1 min 30 s and a single final step at 72°C for 10 min.

Preliminary tests of restriction endonucleases were carried out for 30 individuals drawn from 6 populations (PUA, TOR, MAL, FIR, OST, PIZ). Of the ten restriction enzymes surveyed (*Hae*III, *Rsa*I, *Sau*3a, *Dra*I, *Eco*RI, *Mae*II, *Alu*I, *Ssp*, *Stu*I, *Xho*I), the six which showed restriction sites polymorphism (*Hae*III, *Rsa*I, *Sau*3a, *Dra*I, *Mae*II, *Alu*I) were retained and used to screen all remaining samples.

A volume of 17.3 µl of PCR product was digested over night with 5 units of enzyme following manufacturer's instructions. Restriction fragments were separated on 3% agarose gel, stained with 0.5 µg/ml Ethidium Bromide and visualized under UV light.

Haplotype sequences were obtained using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. The sequencing chromatograms were analysed with the program CHROMAS (Technelysium Pty Ltd, Australia). Alignments were carried out using the software CLUSTALX 1.81 (Thompson *et al.*, 1997).

The null hypothesis of constant substitution rate was tested by means of Tajima's relative rate test (1993) as implemented in MEGA 2.1. Pairwise sequence divergence (*p*-distance) and phylogenetic analyses were computed using the software PAUP* 4.0b10 (Swofford, 2003). Phylogenetic trees were inferred using Neighbour-Joining (NJ) and Maximum Parsimony (MP) analyses. Unweighted MP trees were obtained using the exhaustive search. The reliability of the NJ tree, computed with Kimura 2-parameters distances (K2P; Kimura, 1980), was evaluated by the bootstrapping method with 1,000 pseudoreplicates. *Pseudacris regilla* was used as outgroup (GenBank Accession Number: AY363197; Ripplinger & Wagner, 2004).

4.2.3 ESTIMATING F_{ST} AND GENE FLOW FOR COMPARATIVE PURPOSES

Population genetic structure was investigated using F_{ST} among pairs of samples as estimated by the parameter θ of Weir & Cockerham (1984) using the software FSTAT 2.9.3 (Goudet, 2001). The significance of the θ estimates was assessed by 1000 permutations, and the Bonferroni correction for multiple simultaneous tests (Rice, 1989) was applied.

Indirect estimates of gene flow (*Nm*) among populations were obtained from *F*-statitics (Wright, 1951) as $Nm = (1 - F_{ST})/(4 F_{ST})$. This estimate assumes the infinite-island model of population structure and gene flow (Wright, 1951). Although the majority of real populations probably violate these assumptions (Whitlock *et al.*, 1999), this estimate is still considered a useful tool for comparative purposes (e.g. Neigel, 2002; Lampert *et al.*, 2003; Monsen *et al.*, 2003) and will therefore be used for this aim.

4.3 RESULTS

4.3.1 ALLOZYMES

Eight out of the twenty-three loci surveyed (*6Pgdh, Gapdh, Sod-2, Ck, Adk, Gpi, Pt-2* and *Pt-3*) were monomorphic for the same allele in all populations.

Eight out of 174 tests for HW equilibrium were significant ($P < 0.05$). However, they did not concern specific loci or geographic locations, and were not significant after the Bonferroni correction.

Populations of the Italian treefrog close to the area of parapatry with the European species *H. arborea* (UDI, MAL, SCH, CON and PRE) showed alleles of this species (*Idh-1*^{93}, *Sod-1*^{84}, *Aat-2*^{90}, *Pep-C1*^{108}, *Pep-C2*^{95}, *Pep-C4*^{110} and *Ada*^{93}; Nascetti *et al.*, 1995) introgressed with frequencies varying across loci and populations. The contact zone between these sister species and patterns of introgression are being analyzed in another paper, and will therefore not be further considered here.

Five loci (*Mdhp, Idh-2*, *Aat-2*, *PepC-2* and *Ca3*) showed marked differences in alleles frequencies among populations located alternatively south and north (hereon group-S and group-N respectively) of the northern Apennine (Figure 2). The allele *Mdhp*^{104}, the most common allele at locus *Mdhp* in almost all populations of the group-N, is only present in four populations of the group-S with frequencies never above 0.09, whereas the loci *Idh-2*, *Aat-2*, *PepC-2* and *Ca3* showed alleles found in almost all northern populations that are observed only in one (*Aat-2*^{85} and *PepC-2*^{90}) or two (*Idh-2*^{110} and *Ca3*^{110}) southern populations (see Figure 2).

Nei's (1972) standard genetic distance (D_{NEI}) between populations ranged from 0.00 and 0.11, with the highest value observed between SCH (group-N) and LAR (group-S) and between TOR (group N) and several populations from group-S. Populations close to the northern side of the northern Apennines (MAG, BAG, SLO) showed lower values of D_{NEI} if compared with populations from group-S (D_{NEI} [± standard deviation] being on average 0.02 [± 0.01], 0.03 [± 0.01] and 0.02 [± 0.01] respectively) than if compared with populations from group-N (D_{NEI} being on average 0.04 [± 0.01], 0.07 [± 0.02] and 0.06 [± 0.02] respectively). Between group-N and group-S mean D_{NEI} was 0.07 (± 0.02), whereas within each group it was 0.02 (± 0.01) and 0.01 (± 0.01) respectively.

A phenogram based on a UPGMA (Figure 3) grouped samples into two main clusters corresponding to group-N and group-S as defined above. The only exception to this geographical pattern are the three samples close to the northern side of the Apennines (MAG, BAG, SLO) which are instead included in the southern cluster. Since all other nodes in the UPGMA phenogram receive little bootstrap support (< 70%), additional inferences can't be made with accuracy from the UPGMA phenogram.

The first two principal components resulting from PCA (scatterplot shown in Figure 4) cumulatively accounted for 80.3% of the total genetic variance, although only the first PC was significant ($P < 0.05$). The first principal component explained 72.8% of the total variance and discriminated between the same two groups of samples as observed in the UPGMA tree. Nevertheless, along this PC sample from MAG was intermediate among these two groups.

The level of differentiation among all populations was strong and highly significant (θ=0.311; $P < 0.001$). Values of pairwise θ among samples for which also mitochondrial data were collected are given in Table 3. Observed values of θ within each group suggested a higher level of population differentiation within group-S than within group-N (average θ was 0.126 [± 0.080] and 0.046 [± 0.038] respectively). The sample from MAG shows lower values of θ with respect to samples from group-S than when compared to samples from group-N. However, it is the "southern" sample that shows the lowest values of θ in comparisons with samples from group-N (never exceeding 0.295). The sample from BAG (group-S)

shows the highest values of θ of the intra-group comparisons. In fact, in three cases (when compared to GIB, GAR and OST) it shows values above 0.420, which are of the same degree as the values observed in the inter-group comparisons. When excluding these two populations, average θ between groups was 0.439 (± 0.087), a value that under an infinite islands model (Wright, 1951) leads to an estimate of the average between-groups gene flow of Nm=0.319.

Estimates of population genetic variability are given in Table 4. The unbiased estimate of expected heterozygosity (H_E) varied from 0.016, observed at samples from GAR and LAT, to 0.140 observed at SCH. Mean values of H_E for group-N was 0.112 (± 0.020), whereas for group-S it was 0.049 (± 0.022), values which differ significantly (Mann-Whitney tests: $U_{(n=27;\ d.f.=1)} = 3$; $P < 0.01$). The highest values for H_E were observed in a group of populations close to Eastern Prealps (populations MAL, SCH, CON and PRE). Other measures of genetic variability (observed heterozygosity, mean number of alleles per locus and percentage of polymorphic loci) show the same geographical pattern as H_E.

4.3.2 MTDNA

Five composite RFLP-haplotypes were found over all samples. One of these (a1) was observed only in one individual from MAL, a population close to the hybrid zone between *H. intermedia* and *H. arborea*. It was also observed in all samples studied of *Hyla arborea* from Germany (Verardi, unpublished data). In all samples located south of the northern Apennines the only haplotype observed was s1 (see Figure 2). It was also the only haplotype found at VER and SLO, while in samples from BAG and MAG it was observed at frequencies of 0.8 and 0.5 respectively. In all the other northern populations n1 was the most common haplotype, with frequencies varying from 0.73 (at BAV) to 1.0 (the majority of samples). Two other haplotypes were found among samples of the group-N, neither exceeding 0.22: n2, which was observed at BAV and POR, and n3 at MAG, PUA and BAV.

Sequences of 332 bp corresponding to 16347 (5'end) and 16678 (3' end) of the *Xenopus laevis* mitochondrial genome (Roe *et al.*,1985) were obtained from two individuals for each RFLP-haplotype (GenBank accession numbers: AY093690, AY093692- AY093695).

The NJ tree illustrating relationships among the haplotypes found is given in Figure 5 (the MP tree has an identical topology, hence is not shown). Average sequence divergence (*p*-distance) among *H. arborea* haplotype (a1) and *H. intermedia* haplotypes (n1, n2, n3 and s1) was 14.23%. Among *H. intermedia* haplotypes thirty-two variable sites were observed: 31 in third position and one in second position. No aminoacidic replacement was found. Sequence divergences between haplotypes n1, n2 and n3 ranges from 1 to 3 base pairs, all transitions in third position. The most differentiated haplotype was s1, with an average 9.04% of sequence divergence with respect to the other *H. intermedia* haplotypes.

To assess the linearity of accumulation of substitutions over time we carried out a total of 10 Tajima (1993) tests among all possible pairs of haplotypes, using a previously published sequence of *Pseudacris regilla* as an outgroup. The null hypothesis of constant substitution rates could not be rejected based on those tests (all $P > 0.05$).

The pattern of population differentiation was very strong and significant, with a value of θ overall samples being 0.777 ($P < 0.01$). Pairwise θ estimates between population pairs (Table 4) showed the existence of two groups of populations identical to those evidenced with allozymes. When excluding the samples from BAG and MAG (see below), very weak population differentiation was found within each group, with average θ as low as 0.000 (± 0.000) and 0.032 (± 0.061) for group-S and group-N respectively. On the other hand, average θ between the two groups was as high as 0.950 (± 0.084), which under the infinite islands model (Wright, 1951) gives an estimate of the between-groups gene flow of Nm= 0.013. Populations from BAG and particularly MAG (i.e. the only two where both southern and northern haplotypes were found to be co-present) showed intermediate values of θ when compared either to populations from group-S or to those from group-N.

4.4 DISCUSSION

The study of the genetic variation at 23 nuclear loci and a fragment of the mitochondrial cytochrome *b* gene have clearly shown the existence of a remarkable genetic structuring within the Italian treefrog. This appeared mainly attributable to differences between two geographically coherent groups of populations, one comprising those located north of the northern Apennines, the other those located south. Populations closest to the northern side of this mountain chain was genetically intermediate between the two groups.

Although substantially concordant in depicting spatial pattern of genetic variation, allozymes and mtDNA led to markedly discrepant estimates of the pattern of genetic divergence between the two groups, as well as of the graduality of the intergradation among them. At the mitochondrial level, a sharp phylogeographic break was found, with only two close samples (MAG and BAG) showing the two haploforms co-present. Furthermore, the divergence between the two haploforms was particularly high (*p*-distance=9.04%), resembling that found between several congeneric amphibian species. For instance, Lee *et al.* (1999) observed a sequence divergence of 10.74-10.96% at cytochrome *b* between the Korean *Hyla japonica* and *H. suweonensis*, whereas values of 6.30-10.03% were found by Martinez-Solano *et al.* (2004) among midwife toads of the species *Alytes dickhilleni*, *A. maurus*, *A. muletensis* and *A. obstetricans*. By contrast, genetic divergence at allozymes resulted weaker (D_{NEI}=0.07), and well below the levels usually observed among congeneric amphibian species (e.g. Avise & Aquadro, 1982). By applying Nei's (1975) formula to allozyme data ($t_{(Myr)}=5\cdot10^{6}\cdot D_{NEI}$), the divergence between the two population groups would be dated to 350,000 years. By contrast, the crude estimate of 2% sequence divergence per million years for vertebrate mtDNA (Brown *et al.*, 1979; Johns & Avise, 1998) would lead to an estimate of the divergence time of about 4.5 million years. Even allowing for the vagaries of molecular clocks (e.g. Ayala, 1997 & 1999; Penny, 2005), the two time estimates derived from mtDNA and allozyme data appear largely discrepant. Also the intergradation between the two groups of populations appeared less sharp at allozyme than at mitochondrial level. Samples from SLO and FIR, both clustered within the southern group and sharing the single

'southern' haplotype s1, showed 'northern' nuclear alleles, with low frequencies, at all but one of the differentiated loci. These are also the samples from the southern group that show the lowest genetic differentiation compared to the northern Apennine samples MAG and BAG. Finally, the discrepancy between the two kind of markers is also strikingly evident from pairwise estimates of population subdivision, as measured by conventional *F*-statistics and, consequently, by the F_{ST}-based gene-flow estimates. In fact, given that the mitochondrial genome is haploid and maternally inherited, estimates of gene-flow are expected to be fourfold lower at mtDNA than at nuclear markers, whereas our results indicated a Nm estimate 25 times higher at nuclear than at mitochondrial level.

At least three distinct hypotheses can be made to attempt an explanation of such discrepancies: (i) deviations from expectations under neutral evolution because of forces acting on individual markers, such as balancing selection on allozymes and/or selective sweeps on mtDNA; (ii) past fragmentation followed by secondary-contact through a sex-biased gene-flow; (iii) origin of the southern group through colonization from northern areas, associated with a founder effect, and retention of ancient polymorphisms at allozymes within the northern group.

Balancing selection favours the maintenance of similar allele frequencies, leading to patterns of population subdivision, as measured by conventional *F*-statistics, which should be much less apparent (Schierup *et al.*, 2000). Nevertheless the footprint of natural selection on multiple loci depends on the intensity of selection and the recombination rate among loci (Broughton & Harrison, 2003). Therefore, to play a role in our case study, either this phenomenon would have to be applied simultaneously to several protein loci, or the recombination rate among them would have to be very low, two highly unlikely occurrences. On the other hand, the selective sweep resulting from directional selection on mtDNA could have led to fixation of distinct new haplotypes within one or both of the two groups of population, leading to higher θ-values at mtDNA level. However, this would not alter the amount of sequence divergence among haplotypes, so this phenomenon cannot account for the large discrepancy in the estimates of divergence between the two groups. Therefore, the scenario (i) of differential constraints on marker

evolution, leading to deviations from expectations under neutral evolution, appears on the whole unlikely, and is thus rejected.

The influence of the northern Apennines in shaping patterns of distribution and differentiation among related species or groups of population within them, has been pointed out for several taxa (e.g. Bianco, 1994; Di Giovanni *et al.*, 1998; Stefani *et al.*, 2004). Since the Italian treefrog is a relatively thermophylic species, with more than 90% of its populations located under 500m a.s.l. (e.g. Mazzotti *et al.*, 1999), it appears very likely that this mountain chain has played the role of a barrier to dispersal in this species along the north-south axis. Following the scenario (ii), this mountain chain could have acted as an extrinsic barrier to gene flow, by separating the two population groups, most probably during glacial maxima, when scattered glaciers were present at high altitude (Cremaschi, 2003a & 2003b). This phase would have been followed by a secondary contact, during subsequent interglacials, that re-established gene-flow. However, to generate the observed patterns and discrepancies, this gene flow would be strongly male-biased and directional (mainly from south to north, see Figure 2). Unlike mammals and birds, for which sex-biased gene-flow has been largely documented (e.g. Greenwood, 1980; Helbig *et al.*, 2001; Crochet *et al.*, 2003; Johnson *et al.*, 2003; Baker *et al.*, 1998), much less is known about amphibians (Goudet *et al.*, 2002). Because it is polygynous, the male is expected to be the dispersing sex in frogs (Lampert *et al.*, 2003). For what is at our knowledge, case studies concerning anuran amphibians and requiring male-biased gene-flow as a possible explanation have been reported for only three species: *Rana cascade* (Monsen & Blouin, 2003), *Physalaemus pustulosus* (Lampert *et al.*, 2003) and *Bombina bombina* (Szymura *et al.*, 1985). In the case of the Italian treefrog, a strong male-biased gene-flow from southern to northern areas could have led to extensive introgression at differentiated nuclear loci, giving rise to the general geographic pattern of variation at allele frequencies (see Figure 2), whereas inter-locus variance (Lewontin & Krakauer, 1973; Baer, 1999 and reference therein) could easily account for differences among them. However, the geographic distribution of the genetic variability is hardly explained by this hypothesis alone. In fact it would imply such a geographically asymmetrical gene-flow, and we do not know of ecological or biogeographic features that could underlie this pattern, which appears unlikely.

The alternative hypothesis (iii) of persistent ancient polymorphisms at allozymes within the northern group and the origin of the southern group through colonization from northern areas, could also account for the observed pattern of geographical distribution genetic variation. Because ancestral polymorphisms can be retained, a large historical population size should be assumed (e.g. Ting *et al.*, 2000). Present knowledge of palaeoenvironments in northern Italy allows this assumption (e.g. Montuire & Marcolini, 2002; Cattani, 2003). In fact, during glacial periods, marine transgressions led to a southward migration of the coastal line, till 300 Km south-east of its present location (Cremaschi, 2003b), allowing the formation of freshwater environments where large populations could have persisted (e.g. Miola *et al.*, 2003). By contrast, during interglacials, when the coastal line migrated northward following marine regression, large populations could have persisted in the entire Pò river plain (e.g. Ravazzi & Strick, 1999; Amorosi *et al.*, 1999; Cattani, 2003). The colonization of southern habitats may have occurred during these latter periods, with the establishing of more favourable climatic conditions that allowed treefrogs to cross the northern Apennines. Moreover, the discovery of treefrog fossils from the Early Pleistocene (late Villafranchian) of Apricena (Apulia, southern Italy; Delfino & Bailon, 2000) suggests that such colonization would have pre-dated this period. A founder effect accompanying this event could explain the observed reduced genetic variability of southern populations with respect to populations of the northern group (on average less than half). A reduced historical population size in peninsular Italy may also have been favoured by the more fragmented distribution of lowland habitats in this area.

The two hypotheses (ii) and (iii) are not completely mutually exclusive. Furthermore, each one gives a more plausible explanation for particular aspects of the dataset: the geographical distribution of genetic variability is best explained by hypothesis (iii), well supported by paleoecological and biogeographic information, whereas the patterns of variation among populations closest to the northern Apennines may well have been generated by a secondary contact coupled with a sex-biased dispersal in favour of males.

Therefore, an intermediate scenario between (ii) and (iii) appear the most likely. Within the areas located north of northern Apennines, the maintenance of a

large historical population size would have allowed for the retention of ancestral polymorphisms, which may be responsible for much of the genetic variability found in this area. The areas south of the northern Apennines would have been colonized by the northern populations before the Early Pleistocene, an event accompanied to a founder effect leading to the observed reduced genetic variability in populations from peninsular Italy. The intervening of unsuitable conditions at medium to high altitude in the northern Apennines during subsequent pleniglacials would have made this mountain chain an efficient barrier to dispersal, leading to prolonged isolation between the two population groups. More recently, the re-establishing of climatic conditions favorable to crossing this geographic barrier would have restored some (male-biased) gene-flow between them, and the formation of a secondary contact zone in the area located slightly north of the northern Apennines.

4.5 CONCLUSIONS

In this paper we have presented a case of striking discrepancy between nuclear and mitochondrial markers in depicting the pattern of population differentiation between two groups of populations within the Italian treefrog. The most plausible scenario accounting for the observed pattern of differentiation and the discrepancy among markers appears to imply several historical events: a) the retention of ancestral polymorphisms within the northern group, favoured by a large historical population size; b) the origin of the southern group through colonization from northern areas before the late Villafranchian; c) the isolation between the two population groups during subsequent pleniglacials, with the northern Apennine acting as a barrier to dispersal; d) a secondary contact between them, through a male-biased gene flow.

Further investigations will be needed to conclusively validate the above scenario. In particular, in order to understand the relative contribution of introgression following secondary contact or persistent ancient polymorphisms in shaping the observed pattern of genetic variation in northern Italy, further

investigations will be carried out through the analysis of geographical patterns of variation at paternal inherited (Y-linked) markers.

Based on the results of the mtDNA analysis alone, and with a sparser sampling scheme, one could have supposed the existence of two distinct species within the Italian treefrog, with the northern Apennine acting as an extrinsic barrier to gene-flow. Nevertheless, allozyme data and the study of contact populations do not support this hypothesis. On the other hand, allozyme data alone would have suggested the existence of two moderately differentiated groups of population, whose divergence could be only of recent origin. Only the joint study of both nuclear and mitochondrial markers, has made it possible to put in evidence the existence of a more complex evolutionary history. In the face of the extensive use of a single kind of marker in studies of molecular taxonomy and systematics, the case study we have presented here constitutes a serious cautionary note, and a strong argument against the use of single markers and/or genetic distances for identifying species or investigating evolutionary processes at levels other than the single marker studied. This latter point appears of particular relevance for taxa such as amphibians, whose taxonomy and systematic have been so deeply revised in the light of genetic studies (Veight, 1996; Borkin, 1999).

4.6 TABLES AND FIGURES

Table 1. Geographic origin and sample size (n) of the 33 populations of the Italian treefrog studied.

Sampling location	Location reference code	Latitude N	Longitude E	n	
				Allozymes	mtDNA
Corleone	COR	37°49’	13°18’	25	11
Gibilmanna	GIB	37°55’	14°01’	35	12
Gambarie	GAM	38°09’	15°41’	38	8
Pizzo	PIZ	38°44’	16°10’	23	9
Fiumefreddo	FIU	39°20’	16°07’	8	4
Macchia Longa	MLO	39°15’	16°46’	15	-
Lago Remmo	LAR	40°11’	15°52’	21	-
S. Giovanni Rotondo	GAR	41°43’	15°43’	20	4
Lido di Ostia	OST	41°45’	12°20’	11	7
Latina	LAT	41°28’	12°56’	10	-
Roseto	ROS	42°40	13°59’	-	6
San Lorenzo	SLO	43°34’	13°26’	19	19
Firenze	FIR	43°49’	11°28’	16	6
Bagno di Romagna	BAG	43°50’	11°57’	9	9
Magliano	MAG	44°60’	12°5’	24	24
Punta Alberete	PUA	44°30’	12°16’	17	17
Verucchio	VER	43°59’	12°25'	-	7
Langhirano	LAR	44°37’	10°16'	-	5
Cremona	CRE	45°09’	10°01’	15	15
Novara	NOV	45°29’	8°39’	22	25
Torino	TOR	45°07’	7°34’	8	18
C. Ticino	TIC	46°01	8°54’	-	6
Belluno	BEL	46°08’	12°12’	-	7
Cavarzere	CAV	45°08’	12°04’	7	4
Bavaria	BAV	45°34’	12°05’	17	11
San Daniele	SAN	46°10	13°05’	-	12
Cordenons	POR	45°57’	12°38’	16	9
Punta Sabbioni	SAB	45°30’	12°34’	6	-
Brazzacco	UDI	46°05	13°10’	10	-
Malina	MAL	46°05	13°19’	11	6
Schiavetti	SCH	45°49’	13°33’	21	7
Cona	CON	45°54’	13°32’	12	5
Preval	PRE	45°56’	13°33’	15	8

Table 2. Enzymes studied, their code number, encoding loci, buffer systems and staining technique reference.

Enzyme	EEC	Encoding loci	Buffer systems	Staining reference
Malate dehydrogenase	1.1.1.37	*Mdh-1*	5	b
		Mdh-2	5	b
Malate dehydrogenase ($NADP^+$)	1.1.1.40	*Mdhp*	2	d
Isocitrate dehydrogenase	1.1.1.42	*Idh-1*	2	b
		Idh-2	2	b
6-PhosPhogluconate dehydrogenase	1.1.1.44	*6Pgdh*	5	b
Glyceraldehyde-3-phosfate dehydrogenase	1.2.1.12	*Gapdh*	2	d
Superoxide dismutase	1.15.1.1	*Sod-1*	1,2	d
		Sod-2	1,2	d
Aspartate aminotransferase	2.6.1.1	*Aat-1*	5	c
		Aat-2	5	c
Creatine kinase	2.7.3.2	*Ck*	2	b
Adenylate kinase	2.7.4.3	*Adk*	2	d
Aminopeptidase (Leu-Ala)	3.4.1.1	*Pep-C1*	2	e
		Pep-C2	2	e
		Pep-C4	2	e
Adenosine deaminase	3.5.4.4	*Ada*	2	e
Carbonic anhydrase	4.2.1.1	*Ca*	3	e
Mannose phosphate isomerase	5.3.1.8	*Mpi*	3	e
Glucose phosphate isomerase	5.3.1.9	*Gpi*	5	c
Phosphoglucomutase	5.4.2.2	*Pgm-2*	4	a
Unidentified proteins		*Pt-2*	1	b
		Pt-3	1	b

Buffer systems: 1) Discontinous Tris/Citrate (Poulik, 1957); 2) Continous Tris/Citrate (Selander *et al.*, 1971); 3) Tris/Versene/Borate (Brewer and Sing, 1970); 4) Tris/Versene/Maleate (Brewer and Sing, 1970); 5) Phosfate-Cytrate; Harris (1966). Staining tecnique reference: a) Brewer and Sing (1970); b) Shaw & Prasad (1970); c) Selander *et al.* (1971); d) Ayala *et al.* (1972); e) Harris & Hopkinson (1976).

Table3. Pairwise values of θ (Weir & Cockerham, 1984) for the 22 samples of *Hyla intermedia* for which both mitochondrial and allozymes data were collected.

	COR	GIB	GAM	PIZ	FIU	GAR	OST	SLO	FIR	BAG	MAG	PUA	CRE	NOV	TOR	CAV	BAV	POR	MAL	SCH	CON	PRE
COR	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	**0.156	*0.230	**0.816	**1.000	**1.000	**1.000	**1.000	**0.827	**1.000	**0.884	**1.000	**1.000	**1.000
GIB	*0.056	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	**0.170	*0.239	**0.821	**1.000	**1.000	**1.000	**1.000	**0.834	**1.000	**0.891	**1.000	**1.000	**1.000
GAM	0.010	**0.181	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.107	*0.199	**0.795	**1.000	**1.000	**1.000	**1.000	**0.802	**1.000	**0.858	**1.000	**1.000	**1.000
PIZ	*0.054	*0.084	*0.074	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	**0.125	*0.211	**0.803	**1.000	**1.000	**1.000	**1.000	**0.811	**1.000	**0.868	**1.000	**1.000	**1.000
FIU	*0.092	*0.223	*0.120	0.027	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.125	**0.758	**1.000	**1.000	**1.000	**1.000	**0.750	**1.000	**0.797	**1.000	**1.000	**1.000
GAR	*0.077	≤0.000	**0.209	*0.108	**0.279	x	≤0.000	≤0.000	≤0.000	≤0.000	0.125	**0.758	**1.000	**1.000	**1.000	**1.000	**0.750	**1.000	**0.797	**1.000	**1.000	**1.000
OST	*0.124	0.037	**0.208	*0.092	**0.232	0.051	x	≤0.000	≤0.000	0.087	*0.186	**0.787	**1.000	**1.000	**1.000	**1.000	**0.791	**1.000	**0.847	**1.000	**1.000	**1.000
SLO	**0.146	**0.263	*0.117	**0.165	*0.137	**0.294	**0.262	x	≤0.000	**0.249	**0.289	**0.853	**1.000	**1.000	**1.000	**1.000	**0.871	**1.000	**0.922	**1.000	**1.000	**1.000
FIR	*0.045	**0.155	**0.085	*0.088	*0.068	**0.175	*0.153	0.030	x	0.063	*0.171	**0.779	**1.000	**1.000	**1.000	**1.000	**0.779	**1.000	**0.833	**1.000	**1.000	**1.000
BAG	*0.251	**0.420	0.112	*0.245	*0.185	**0.486	**0.442	0.033	*0.098	x	0.016	**0.584	**0.807	**0.860	**0.827	**0.662	*0.553	**0.750	*0.552	*0.722	**0.685	*0.737
MAG	**0.143	**0.191	**0.159	**0.136	*0.111	**0.188	**0.187	**0.071	0.027	*0.107	x	**0.347	**0.486	**0.552	**0.508	*0.358	**0.302	**0.432	*0.297	*0.409	**0.379	**0.421
PUA	**0.424	**0.438	**0.428	*0.347	**0.320	**0.433	**0.353	**0.351	**0.311	**0.380	**0.177	x	0.112	**0.169	**0.131	≤0.000	≤0.000	*0.062	≤0.000	0.036	≤0.000	0.050
CRE	**0.588	**0.615	**0.566	**0.537	**0.514	**0.619	**0.529	**0.493	**0.454	**0.523	**0.295	*0.087	x	≤0.000	≤0.000	≤0.000	**0.085	≤0.000	**0.167	≤0.000	≤0.000	≤0.000
NOV	**0.494	**0.573	**0.521	**0.492	**0.453	**0.566	**0.481	**0.425	**0.392	**0.398	**0.232	*0.063	0.007	x	≤0.000	≤0.000	**0.152	≤0.000	**0.275	≤0.000	≤0.000	≤0.000
TOR	**0.595	**0.624	**0.506	**0.530	**0.462	**0.640	**0.484	**0.479	**0.425	**0.443	**0.277	0.026	0.001	≤0.000	x	≤0.000	**0.108	≤0.000	**0.204	≤0.000	≤0.000	≤0.000
CAV	**0.524	**0.590	**0.431	**0.455	**0.396	**0.626	**0.480	**0.376	**0.333	**0.347	*0.168	0.018	*0.091	0.017	0.013	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000
BAV	**0.449	**0.487	**0.427	**0.410	**0.363	**0.486	**0.381	**0.358	**0.315	**0.364	**0.188	0.024	0.044	0.023	*0.048	≤0.000	x	0.027	≤0.000	≤0.000	≤0.000	0.014
POR	**0.419	**0.449	**0.357	**0.350	*0.374	**0.478	**0.309	**0.368	**0.320	**0.387	**0.181	0.026	*0.065	0.030	0.036	0.018	≤0.000	x	**0.072	≤0.000	≤0.000	≤0.000
MAL	**0.474	**0.398	**0.322	*0.350	**0.331	**0.491	*0.302	**0.377	**0.320	**0.302	*0.142	0.024	0.004	≤0.000	*0.085	0.060	*0.056	0.027	x	**0.028	≤0.000	**0.051
SCH	**0.489	**0.518	**0.434	**0.454	**0.385	**0.518	**0.417	**0.415	**0.357	**0.379	**0.264	**0.148	**0.150	0.040	*0.090	*0.101	*0.126	*0.107	*0.056	x	≤0.000	≤0.000
CON	**0.531	**0.528	**0.432	**0.447	**0.388	**0.570	**0.424	**0.421	**0.378	**0.376	**0.211	0.049	0.042	0.006	*0.068	0.040	*0.069	*0.080	0.044	*0.083	x	≤0.000
PRE	**0.417	**0.407	**0.382	**0.342	**0.317	**0.427	**0.311	**0.342	**0.293	**0.333	**0.148	≤0.000	*0.073	0.015	*0.067	0.017	0.012	0.012	0.008	**0.092	*0.060	x

Allozymes data are below the diagonal; mitochondrial data are above the diagonal.
*P <0.05 after 1000 permutations.
**Significant after Bonferroni correction.

Table 4. Estimates of genetic variability for sampled populations of the Italian treefrog (standard deviation in parentheses). Samples are encoded as in Table 1.

Sample	A	P_{95}	H_O	H_E
COR	1.2 (0.1)	8.7	0.026 (0.023)	0.034 (0.023)
GIB	1.3 (0.1)	8.7	0.026 (0.013)	0.027 (0.013)
GAM	1.2 (0.1)	13	0.056 (0.036)	0.048 (0.032)
PIZ	1.3 (0.1)	13	0.045 (0.023)	0.056 (0.030)
FIU	1.2 (0.1)	13	0.071 (0.042)	0.059 (0.036)
MLO	1.3 (0.1)	21.7	0.074 (0.036)	0.065 (0.030)
LAR	1.3 (0.2)	13	0.050 (0.030)	0.045 (0.028)
GAR	1.1 (0.1)	8.7	0.011 (0.009)	0.016 (0.010)
OST	1.2 (0.1)	13	0.027 (0.014)	0.037 (0.017)
LAT	1.1 (0.1)	8.7	0.017 (0.014)	0.016 (0.012)
SLO	1.3 (0.1)	13	0.067 (0.032)	0.061 (0.029)
FIR	1.3 (0.1)	26.1	0.070 (0.028)	0.067 (0.027)
BAG	1.2 (0.1)	17.4	0.048 (0.023)	0.052 (0.025)
MAG	1.3 (0.1)	26.1	0.089 (0.038)	0.096 (0.036)
PUA	1.4 (0.1)	30.4	0.113 (0.039)	0.108 (0.036)
CRE	1.2 (0.1)	17.4	0.049 (0.025)	0.077 (0.035)
NOV	1.2 (0.1)	17.4	0.091 (0.041)	0.090 (0.040)
TOR	1.4 (0.1)	39.1	0.121 (0.039)	0.129 (0.039)
CAV	1.3 (0.1)	30.4	0.119 (0.042)	0.110 (0.038)
BAV	1.5 (0.1)	34.8	0.113 (0.041)	0.106 (0.034)
POR	1.3 (0.1)	26.1	0.088 (0.034)	0.092 (0.035)
SAB	1.2 (0.1)	21.7	0.087 (0.044)	0.101 (0.041)
UDI	1.4 (0.1)	39.1	0.072 (0.026)	0.099 (0.033)
MAL	1.6 (0.1)	34.8	0.134 (0.041)	0.138 (0.038)
SCH	1.4 (0.2)	30.4	0.132 (0.049)	0.140 (0.049)
CON	1.4 (0.1)	34.8	0.125 (0.046)	0.134 (0.043)
PRE	1.6 (0.1)	47.8	0.129 (0.031)	0.131 (0.035)

A, mean number of alleles per locus; P_{95}, percentage of polymorphic loci (95% criterion); H_O, observed heterozygosity; H_E, expected heterozygosity (Nei's 1978 unbiased estimate).

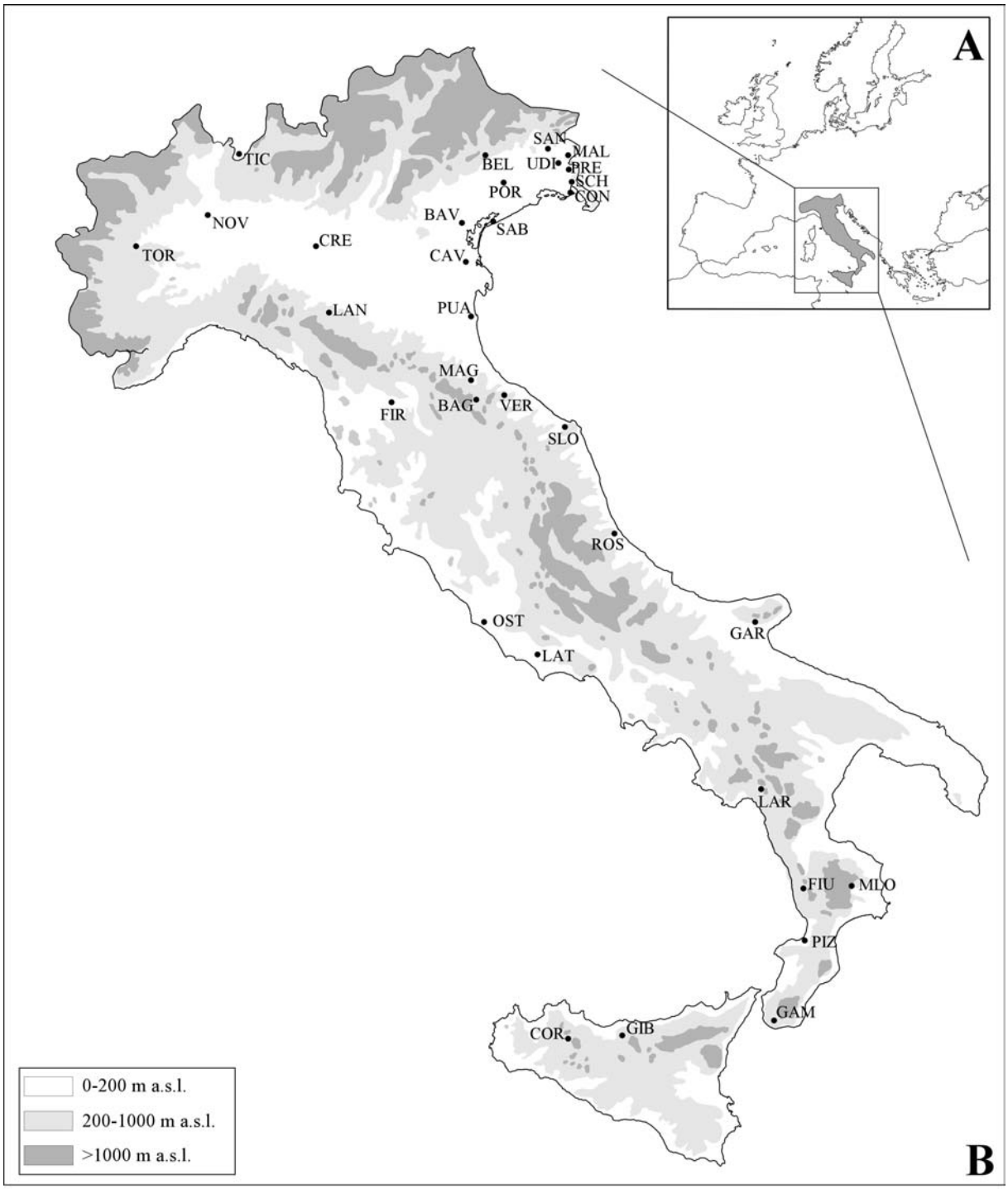

Figure 1. Geographic range of *Hyla intermedia* (A) and geographic location of the 33 samples studied (B). Samples are encoded as in Table 1.

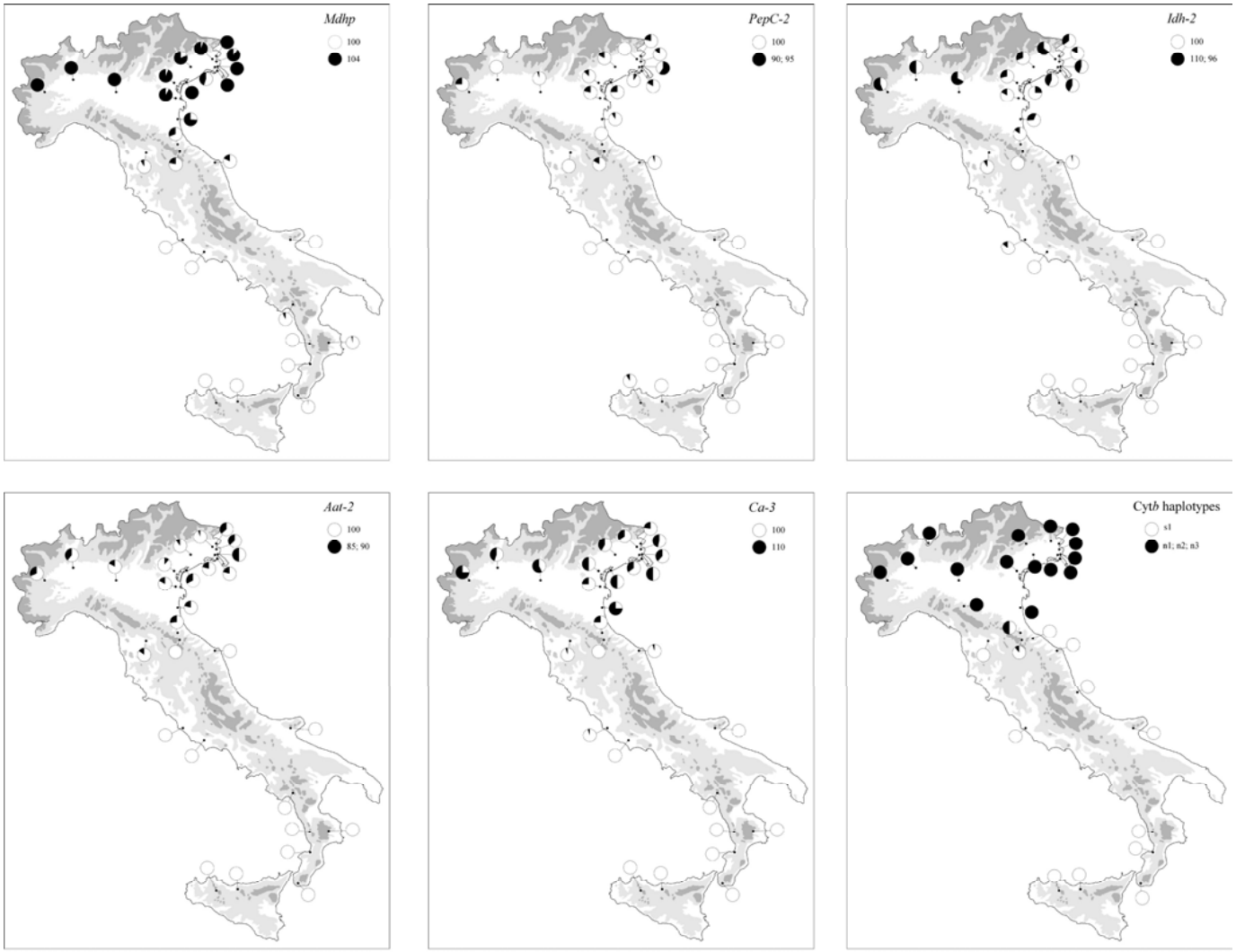

Figure 2. Pie-diagrams showing the geographic variation of allele frequencies at loci *Mdhp, PepC-2, Idh-2, Aat-2, Ca-3* and of haplotype frequencies at the mitochondrial gene encoding for the cytochrome *b*.

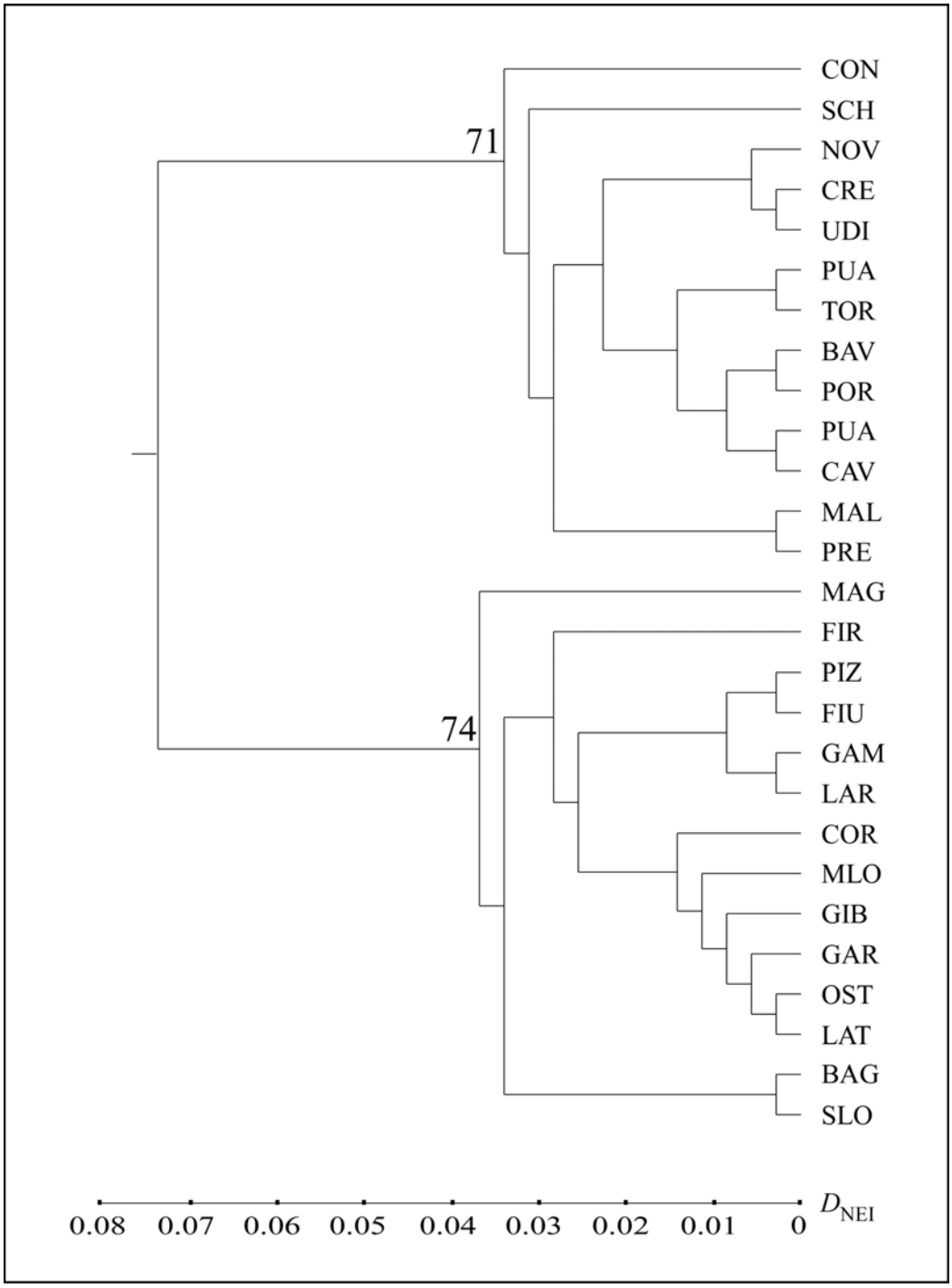

Figure 3. UPGMA phenogram of the populations sampled of the Italian treefrog, based on Nei's (1972) standard genetic distance (D_{NEI}). Bootstrap values > 70% after 1000 pseudoreplicates are shown.

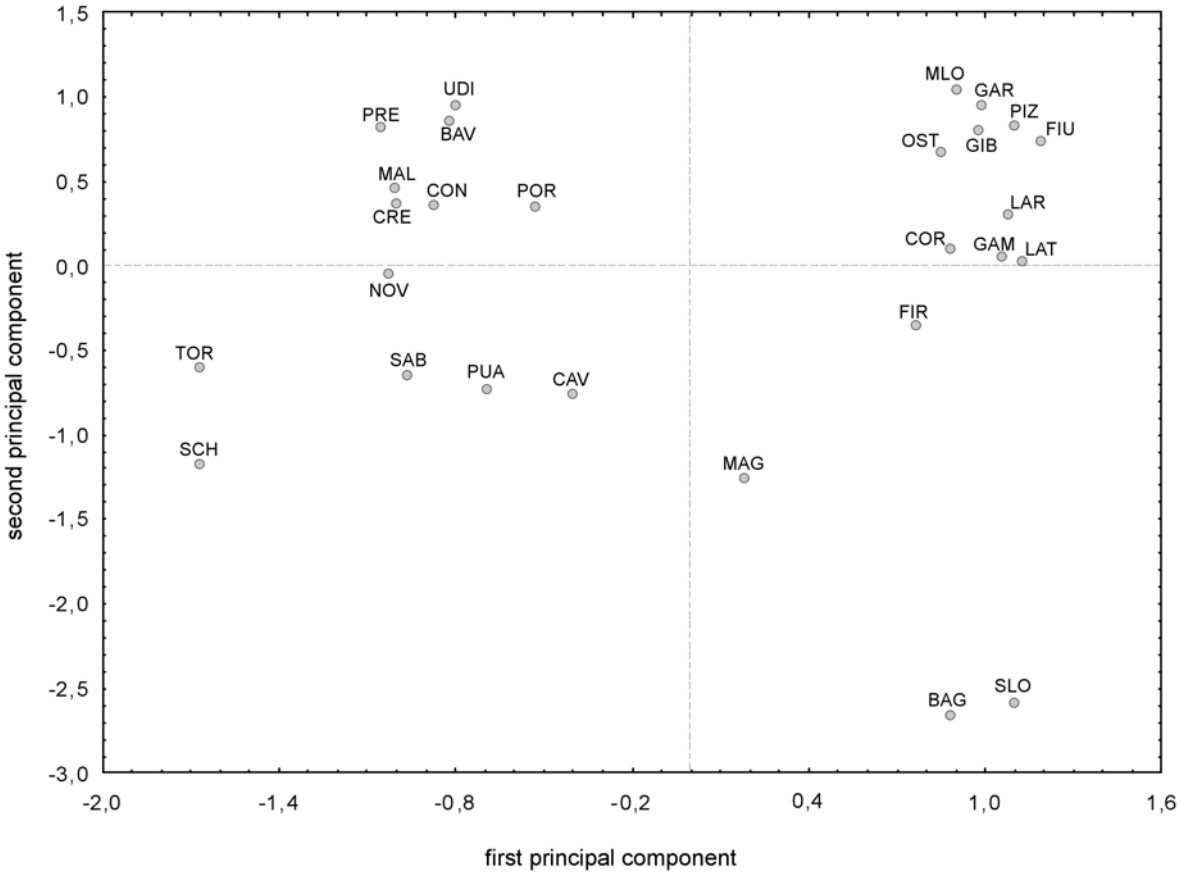

Figure 4. Principal component analysis of allele frequencies among the studied samples of the Italian treefrog. The horizontal axis is significant ($P < 0.01$), whereas the vertical is not. Samples are encoded as in Table 1.

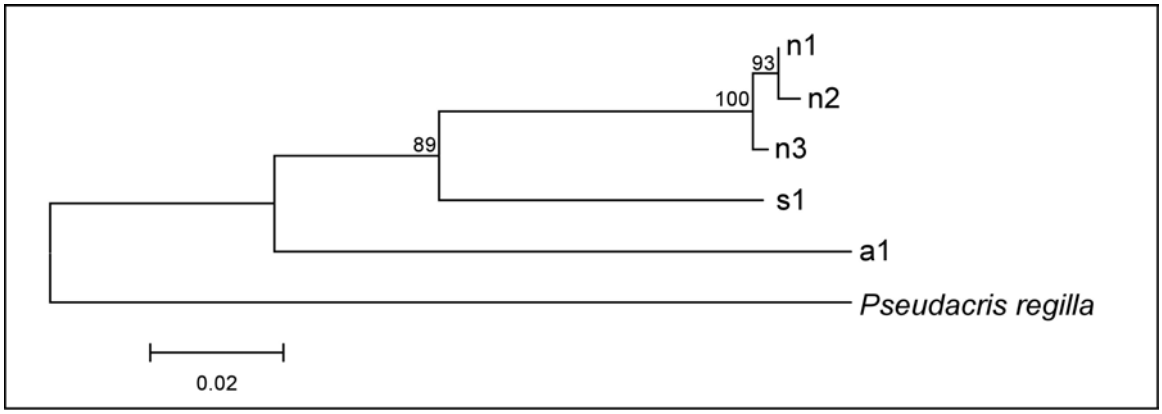

Figure 5. Neighbour-joining tree based on K-2-P genetic distances among the cytochrome *b* haplotypes found within the studied samples of *Hyla intermedia*. Bootstrap support over 1000 replicates are shown at the nodes. Pseudacris regilla (GenBank Accession Number: AY363197; Ripplinger & Wagner, 2004) was used as outgroup.

5. GENETIC DIVERSITY AND PHYLOGEOGRAPHY OF THE APENNINE YELLOW-BELLIED TOAD *Bombina pachypus* (ANURA: DISCOGLOSSIDAE), WITH IMPLICATIONS FOR CONSERVATION.

DANIELE CANESTRELLI, ROBERTA CIMMARUTA, VERA COSTANTINI & GIUSEPPE NASCETTI

ABSTRACT

Genetic variation was investigated in 17 populations of the Italian endemic Apennine yellow-bellied toad using both mitochondrial (598bp of the cytochrome *b* gene) and nuclear (21 allozyme loci) markers. Populations from central Calabria (southern Italy) showed the highest levels of intrapopulation genetic variation, whereas samples located north of this region were nearly lacking in variation. This appears to be a typical pattern of 'southern richness and northern purity', usually attributed to the prolonged population stability within southern refugia coupled with the loss of variation during post-glacial northward expansion. However, the overall pattern of genetic variation observed has a strong geographical component, suggesting two Calabrian plains, Catanzaro and Crati-Sibari, as historical barriers to dispersal separating three population groups. These findings cannot be explained by the prolonged stability of southern populations alone and suggest that the southern richness has been at least in part shaped by allopatric differentiation within the refugial range, followed by intermixing of previously differentiated lineages. From a conservation standpoint, Calabria is the major genetic diversity reservoir for this species, thus deserving particular conservation efforts. Furthermore, although the low intrapopulation genetic variation outside Calabria appears to be of clear historical origin, a current reduction of gene flow indicated by the nested clade analysis and the finding of three private haplotypes each fixed in a single sample from the same river drainage basin, suggests that anthropogenic factors have also contributed.

KEYWORDS: *Bombina pachypus*; allozymes; mtDNA; glacial refugia; genetic diversity; Italy.

5.1 INTRODUCTION

Climatic oscillations during the Pleistocene have greatly affected the pattern of distribution of many species in the Western Palaearctic region, as well as their demographic history and patterns of population genetic differentiation (for extensive reviews see Avise, 2000; Hewitt 2004a & 2004b). The ranges of many organisms were fragmented during glacial maxima, with geographic isolates persisting in glacial refugia in the Mediterranean or western Asia, and particularly in the southern portions of the Mediterranean peninsulas of Italy, Iberia, and the Balkans (but see Stewart & Lister, 2001 and Deffontaine *et al.*, 2005 and references therein). Following environmental amelioration during interglacials, several taxa underwent a northward range expansion from distinct southern refugia, giving rise to recolonizations of northern environments, and in several cases to the establishment of secondary contact zones between previously isolated evolutionary lineages (Taberlet *et al.*, 1998; Hewitt, 1999). One of the major implication of this general scenario is the pattern of "southern richness and northern purity" (Hewitt 1996, 1999 & 2000). That is, the higher population genetic diversity found in the south of the Mediterranean peninsulas (i.e. in the former glacial refugia) compared with populations located further north. Such a pattern was mainly attributed to the progressive loss of genetic variation due to serial bottlenecking during the process of rapid northward range expansions (Hewitt, 1996 & 2000; but see Bilton *et al.*, 1998; Austerlitz *et al.*, 2000; Petit *et al.*, 2003). The high genetic diversity found in the south would therefore be mainly attributed to the prolonged demographic stability of the populations in these areas (Hewitt, 1996). More recently, following the burgeoning amount of phylogeographic studies published regarding the Iberian peninsula, growing emphasis has been placed on an alternative hypothesis to explain such a pattern (Sanz *et al.*, 2000; Guillame *et al.*, 2000; see Gomez & Lunt, 2004 for a

review of evidence). This arises from the finding of a strong population differentiation within this glacial refugium for a wide variety of taxa, suggesting the existence of multiple refugia within the Iberian peninsula. This pattern of "refugia-within-refugia" (Gomez & Lunt, 2004) has important implications in interpreting the distribution patterns of genetic diversity within the southern peninsulas. Firstly, the "southern richness" could have arisen from at least two mutually non-exclusive causes: i) the prolonged demographic stability of populations from these areas (Hewitt, 1996), or ii) the existence of a high geographic structuring of populations due to allopatric differentiation (Sanz *et al.*, 2000; Guillame *et al.*, 2000). Secondly, since the major contribution to the process of northward recolonization is given by populations closest to the northern edge of the species range, if a strong population structure exist the process of genetic diversity loss due to serial bottlenecks during range expansion would start from a subset of the overall southern diversity, and would therefore be more pronounced (Gomez & Lunt, 2004 and references therein). Burgeoning evidence supports this scenario for the Iberian peninsula (e.g. Jaarola & Searle, 2004; Vila *et al.*, 2005).

As to the Italian refugium, a prolonged demographic stability of populations (sometimes coupled with a northward range expansion during interglacials) was assumed as a likely common history for many taxa from this geographic area, by authors emphasizing its role either as glacial refugium and source-area for successive recolonization of northern habitats, either as an area of high endemicity (Hewitt, 1996; Bilton *et al.*, 1998; Petit *et al.*, 2003). However, the possible contribution of a "refugia-within-refugia" scenario still remains substantially unexplored, and more broadly of allopatric differentiation, in shaping the pattern of genetic diversity in the south of the Italian peninsula (but see Santucci *et al.*, 1996; Podnar *et al.*, 2005).

In this paper we present an assessment of the pattern of distribution of genetic diversity and population genetic structure of the Apennine yellow-bellied toad *Bombina pachypus*, as emerging from the analysis of variation at both nuclear (23 allozyme loci) and mitochondrial markers (a 598 bp fragment of the cytochrome *b* gene). This species is endemic to peninsular Italy (a report by Bruno, 1970 placing the species in north-eastern Sicily still needs confirmation), and some authors have suggested that it originated during the Quaternary ice ages, when this species and its

sister taxon *Bombina variegata* reached their glacial refugia in southern Italy and the Balkans respectively (Nascetti *et al.*, 1982; Szymura *et al.* 1985; Szymura, 1993). Preliminary allozyme data also suggested a higher genetic diversity in the southern portion of the range than in the northern one (Nascetti *et al.*, 1982). Therefore, this species appeared a suitable case study to start investigating whether and to what extent the so-called "southern richness" may be attributed to the existence of population subdivisions within the putative refugial area in southern Italy, rather than to prolonged demographic stability, which is the first aim of this paper.

Once regarded as a subspecies of *Bombina variegata*, the Apennine yellow-bellied toad is now considered as a distinct species (after Lanza & Vanni, 1991; Lanza & Corti, 1993) on the basis of both allozyme (Nascetti *et al.*, 1982) and morphological (Vaccaneo, 1931) evidence (but see Veith, 1996; Sarrocco & Bologna, 2000). It breeds in temporary pools and other shallow and unshaded freshwater environments (Lanza, 1983), and is mainly distributed at mid-altitude areas along the Apennine chain (e.g. Caputo *et al.*, 1985; Doria & Salvidio 1994; Mazzotti *et al.*, 1999; Sarrocco & Bologna, 2000). The species conservation status is a cause for concern, and it is protected (still under the name of *B. variegata*) by several international conventions and regional laws (it is listed in the Annex II of the Bern Convention and in the Annexes II and IV of the EU Council Directive for the Conservation of Natural Habitats and of Wild Fauna and Flora). The species range appears fragmented, and several authors have recently suggested that many of its populations could be declining and disappearing, and that the extant populations are often constituted of few individuals (e.g. Caputo *et al.*, 1985; Doria & Salvidio, 1994 and references therein; Sarrocco & Bologna, 2000). Since genetic data are among the most relevant for the assessment of both the species' conservation status and management priorities (for extensive reviews see Avise & Hamrick, 1996; Young & Clarke, 2000; Frankham *et al.*, 2002; Ferrière *et al.*, 2004), in this paper we will also discuss the implications of our results for the conservation of the species.

5.2 MATERIALS AND METHODS

5.2.1 SAMPLING

We collected 161 specimens of *Bombina pachypus* from 17 localities ranging from the northern Apennines to the tip of Calabria. Geographic location of sampling sites and sample size are shown in Table 1 and Fig. 1a. Live specimens were anaesthetized in the field by submersion in a 0.02% solution of MS222 (3-aminobenzoic acid ethyl ester) following the suggestions of Donnelly *et al.* (1994). Tissue samples were obtained through removing 3 to 4 toes (toe-clipping) from each specimen, which was then released in the same collection place. Collected tissue samples were transported to the laboratory in a 75% solution of EtOH (samples 6,8,10 and 13) or in liquid nitrogen containers and then stored at –80 °C until further analyses (all other samples).

5.2.2 DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total DNA was extracted using C-TAB procedure (Doyle & Doyle, 1987). Partial sequences of the mtDNA gene encoding for the cytochrome *b* were obtained through PCR-amplification. Amplification was performed in a volume of 50µl, containing $MgCl_2$ (2.5mM), the reaction buffer (1X; Promega), the four dNTPs (0.2mM each), the two primers (0.2µM each), the enzyme *Taq* polymerase (2 units; Promega) and 2µl of DNA template. Preliminary amplifications were performed using the generic primers L14841 (Kocher *et al.,* 1989) and MVZ16 (Moritz *et al.,* 1992). The sequences obtained with these primers were used to design the specific primers 494BOMOD (5'-GCATGG TGAAATTTTGGCTCC-3') and CYTbBOMOD (5'-CCTAGTAGGTTTGGGGTGAATAT AGC-3'), which were then used to screen all studied specimens. PCR cycling procedure was: 95°C for 5 min followed by 33 cycles of 93°C for 1 min, 52°C for 45 s, 72°C for 1 min 30 s and a single final step at 72°C for 10 min. Sequences were obtained using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. All specimens analysed were double sequenced.

Multiple sequence alignments were made using CLUSTALX (Thompson *et al.*, 1997) and checked by eye.

5.2.3 MTDNA DATA ANALYSIS

Haplotype network estimation and Nested Clade Phylogeographical Analysis (NCPA) - The genealogical relationships between the haplotypes found were represented by a network. This form of data representation is regarded as more appropriate than traditional tree-building methods when analyzing intraspecific differentiation, as we were doing, because it does not force data into a bifurcating pattern, but allows for persistent ancestral nodes, multifurcations and reticulations (Posada & Crandall, 2001). The haplotype network was estimated using the statistical parsimony algorithm described by Templeton *et al.* (1992), and implemented by the software TCS 1.13 (Clemment *et al.*, 2000). In order to investigate the role of historical events and population structure in shaping the present geographical pattern of variation at mtDNA, we conducted a NCPA. With this aim, the haplotype network was converted into a nested series of clades according to the rules given in Templeton *et al.* (1987) and Templeton & Sing (1993). The software GEODIS 2.0 (Posada *et al.*, 2000) was used to calculate the geographical center of haplotypes and clades as well as, for each of them, to test for the significance of two distance measures: the clade distance (D_C) and the nested clade distance (D_N). D_C is the average geographical distance between all members of a clade and its geographical center, whereas D_N is the average distance between the members of a clade and the geographical center of the clade within which that clade is nested. Therefore, the first measure represents an estimate of how geographically widespread a clade is, whereas the second is a measure of its geographical isolation with respect to the other clades with which it is nested (Templeton *et al.*, 1995). Results of the analysis were interpreted using the latest published version of the inference key (Templeton, 2004). At the highest nesting level, tip/interior status was assessed by outgroup rooting. With this aim, a Maximum Parsimony analysis (heuristic search, 50% majority rule; data not shown) was carried out with *Bombina variegata variegata* as outgroup (GenBank Accession Number: DQ320161). To test for a secondary contact between the lineages detected, we also performed the supplementary test to the NCPA proposed by

Templeton (2001). For each nesting level, the average pairwise distance between the geographical center of haplotypes and clades was calculated for all populations, and then plotted in order of increasing latitude and clade level.

Genetic diversity and population structure – The extent of the mtDNA diversity at population level was evaluated by estimating nucleotide (π) and haplotype diversity (h) as described by Nei (1987), and implemented in the software DNASP 4.0 (Rozas *et al.*, 2003). In order to detect signatures of past population growth, the same software was also used to calculate the statistics F_S (Fu, 1997) and R_2 (Rasmos-Onsins & Rozas, 2002), which in a recent study appeared the most powerful for the detection of recent demographic expansions (Ramos-Onsins & Rozas, 2002). The statistical significance of these statistics was evaluated by coalescent simulations (10 000 replicates).

Genetic differentiation between populations was assessed by estimating pairwise values of F_{ST}, and the associated significances (by 100,000 permutations), using the software ARLEQUIN 2.0 (Schneider *et al.*, 1999). This software was also used to perform an Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 1992) in order to partition the total genetic variance into its components among population groups, among populations within groups and within populations (significances assessed by 100,000 permutations). For this analysis, population groups were defined according to the major phylogeographic breaks indicated by the NCPA. Both the pairwise F_{ST} and AMOVA analyses were carried out incorporating information about divergence among haplotype pairs. With this aim, they were run using Tamura-Nei (1993) genetic distance, which is the best approximation available in ARLEQUIN of the best-fit model of sequence evolution (HKY), selected through the Akaike Information Criterion as implemented by the program MODELTEST 3.6 (Posada & Crandall, 1998).

In order to ascertain the existence of a migration-drift equilibrium at a regional scale, we used the approach proposed by Hutchinson & Templeton (1999). Under a stepping stone model of population structure (which is consistent with the natural history of the study species), the relative importance of these forces in shaping the pattern of population structure can be evaluated by predictable and contrasted patterns of relationship between genetic and geographical distances, as

well as by the scattering degree of genetic over geographic distance. Under equilibrium conditions both genetic distance and its variance are expected to increase with increasing geographic distance between populations, giving rise to positive and monotonic relationships. The lack or weakness of one or both of these relationships is instead indicative of a lack of regional equilibrium between gene flow and drift. Because gene flow is an homogenizing force, when it is more influential then drift (case II of Hutchinson & Templeton, 1999) a pattern reflecting panmixia is expected, with no relationships between genetic and geographic distance and little variance in estimates of genetic divergence. This variance is instead expected to be large when genetic drift is strong, relative to gene flow, as in cases where habitat discontinuity has led to extensive population isolation (case III of Hutchinson & Templeton, 1999). The relationship between geographical (in km) and genetic (F_{ST}) distances was evaluated through a Mantel test (with 10,000 permutations), as implemented by the software IBD 1.52 (Bohonak, 2002). The reduced major axis regression was used to evaluate the strength of the relationship, and to calculate regression statistics. Moreover, to determine whether the variance of the F_{ST} estimates increases with the geographic distance separating population pairs, the residuals obtained from the regression of the geographic distances vs. F_{ST} estimates were correlated with the geographic distances (following Hutchinson & Templeton, 1999), using the same statistical procedure as above.

5.2.4 ALLOZYME ELECTROPHORESIS

Standard horizontal starch gel (10%) electrophoresis was performed in order to screen the samples for their allozyme variation. The following enzyme systems (16) and loci (21) were resolved: Glycerol-3-phosphate dehydrogenase (*G3pdh;* EC 1.1.1.8), Lactate dehydrogenase (*Ldh-1* and *Ldh-2;* EC 1.1.1.27), Malate dehydrogenase (*Mdh-1* and *Mdh-2*; EC 1.1.1.37), Malate dehydrogenase $NADP^+$-dependent (*Mdhp-1* and *Mdhp-2;* EC 1.1.1.40), Isocitrate dehydrogenase (*Icdh-1* and *Icdh-2;* EC 1.1.1.42), 6-Phosphogluconate dehydrogenase (*6Pgdh;* EC 1.1.1.44), Superoxide dismutase (*Sod;* EC 1.15.1.1), Aspartate transaminase (*Aat-1* and *Aat-2;* EC 2.6.1.1), Creatine kinase (*Ck;* EC 2.7.3.2), L-LeucylGlycylGlycine Peptidase (*Pep-B;* EC 3.4.11.23), L-Phenylalanyl-L-proline Peptidase (*Pep-D;* EC 3.4.13.9), Anidrasi

carbonica (*Ca;* EC 4.2.1.1), Aconitase (*Aco;* EC 4.2.1.3), Mannose phosphate isomerase (*Mpi;* EC 5.3.1.8), Glucose phosphate isomerase (*Gpi;* EC 5.3.1.9), Phosphoglucomutase (*Pgm-2;* EC 5.4.2.2). Zymograms were visualized using enzyme-specific staining procedures, following techniques in Harris & Hopkinson (1976) and Richardson *et al.* (1986). Alleles at each locus were designated by their mobility (in mm, standardized conditions) relative to the most common one (*100*) in a reference population (Bagno di Romagna).

5.2.5 ALLOZYME DATA ANALYSIS

Estimates of allele frequencies and population genetic variability (as mean observed heterozygosity, Nei's (1978) unbiased estimate of expected heterozygosity, proportion of polymorphic loci and mean number of alleles per locus) were computed with the software BIOSYS-2 (Swofford & Selander, 1999). Exact significance probabilities for departures from the expected Hardy-Weinberg equilibrium were computed for each locus in each sample.

A hierarchical cluster analysis of populations was carried out using the Neighbour-Joining method (NJ) with Cavalli-Sforza & Edwards (1967) chord distances. The reliability of the NJ tree was assessed by 1,000 bootstrap replicates, obtained with the BOOTDIST option in BIOSYS-2. The consensus NJ tree was then obtained using the subroutines NEIGHBOR and CONSENSE in the software PHYLIP 3.5c (Felsenstein, 1993). Because tree-building methods force populations into a dichotomic branching pattern we also, in order to detect potentially intergraded populations relative to the main lineages, computed a Principal Component Analysis (PCA) of allele frequencies by means of the software PCAGEN 1.2 (Goudet, 1999). The statistical significance of each axis was evaluated over 10,000 randomizations.

The amount of genetic differentiation between populations was investigated by calculating pairwise F_{ST} values as estimated by the parameter θ of Weir & Cockerham (1984), using the software FSTAT 2.9.3 (Goudet, 2001). The significance of the F_{ST} estimates was assessed by 1000 permutations. As for mitochondrial data, the hierarchical partitioning of the genetic variance into its different components was evaluated by an AMOVA analysis (Excoffier *et al.*, 1992), as implemented by the software ARLEQUIN 2.0 (Schneider *et al.*, 1999).

The relationships between geographical and genetic distances were investigated using the same methodological framework as for mtDNA data. The statistical significance of the association between the genetic (F_{ST}) and geographic (in km) distance matrices was assessed through a Mantel test (with 10,000 permutations), whereas its strength as well as regression statistics were assessed by means of the reduced major axis regression as implemented in the software IBD 1.52 (Bohonak, 2002).

5.3 RESULTS

5.3.1 MTDNA

Sequence variation and population genetic diversity – For all the 148 studied specimens we obtained a fragment of 598 base pairs of the mitochondrial cytochrome *b* gene, corresponding to the positions 14286-14884 of the previously published *Bombina bombina* mitochondrial genome (GenBank accession number: NC 006402). Thirteen distinct haplotypes were found (GenBank Accession Numbers: DQ320148-DQ320160), showing a sequence divergence ranging from 0.3% to 1.0% (the overall average being 0.6%). These were identified by thirteen variable positions, with all substitutions being transitions, nine located at the third codon position, three at the second position and one at the first position. Substitution located at the first and second position gave rise to the following four aminoacidic replacements: Ile/Val, Ala/Val, Gln/Arg and Ser/Asn.

Estimates of haplotype (*h*) and nucleotide diversity (π) for each sampled population are given in Table 2. Strikingly, twelve out of seventeen populations analysed showed a complete lack of variability at the mtDNA gene fragment analysed and are all located north of the Crati-Sibari plain (samples 1-10), except for the two southernmost samples (16-17). The only populations showing appreciable mtDNA diversity are those located in central Calabria (samples 11-15). Moreover, both *h* and π were particularly high in samples 13 and 14, located respectively slightly north and slightly south of the Catanzaro plain.

Haplotype network and geographical distribution of haplotypes – The minimum spanning network yielded by the statistical parsimony procedure is presented in Fig. 1b. A reticulation between haplotypes h5 and h7, caused by an inferred haplotype, was removed following the criteria of Crandall & Templeton (1993).

The haplotype showing the highest number of connections in the network was h6, which was found to be restricted to the three populations (12-14) sampled in the geographical area between the Crati-Sibari plain and the Catanzaro plain (later on CS- and CA-plain respectively), where it is also the most common haplotype (42.3% of the specimens analysed from this area). Within this geographic area, five other haplotypes were found (h7, h8, h9, h10 and h11), three of which were never found elsewhere (h7, h8 and h10). Among the populations sampled along the Apennine chain north of the CS-plain (1-10), the most common haplotype was h1, which was fixed in six out of ten populations (2-5, 9-10), but which was never found south of CS-plain. Each of the other four populations sampled north of this area (the sample 1 from the northern Apennines and the three samples from the Volturno river basin 6, 7, 8) showed a single private haplotype fixed. Among the four populations sampled south of the CA-plain (14-17), the haplotype h11 was by far the most common, being shared by 92.7% of the specimens analysed. Of the other three haplotypes observed in this area (each from a single specimen), two were restricted to it, and the third (h9) was shared with an individual sampled north of CA-plain (from sample 13).

Nested Clade Phylogeographical Analysis (NCPA) – Results of the NCPA of geographical distance and the application of Templeton's (2004) inference key are presented in Fig. 2, while the nesting design upon which the analysis is based is presented in Fig. 1b. Both population structure and history appeared to have played a role in shaping the observed geographic pattern of association of haplotypes and clades. For the clade 1-1, comprising all the haplotypes found in (and restricted to) the samples located north of CS-plain, restricted gene flow/dispersal was inferred as the main cause of the geographical association, whereas an event of long distance dispersal has been inferred for the haplotype h2, which is geographically restricted to sample 1, collected on a ridge of the northern Apennines. A contiguous range expansion, on the other hand, was inferred for the clade 1-3, comprising haplotypes

sampled in central Calabria. For the clade 2-2, a range expansion was suggested, either through long-distance colonization possibly coupled with subsequent fragmentation or by past fragmentation followed by range expansion. Three 1-step clades were nested within clade 2-2: the clade 1-2, which comprised a single haplotype (h7) found in one sample only (11), and the clades 1-1 and 1-3 which were distributed respectively north and south of the CS-plain. An inference of long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion, was also made at the level of the total cladogram, where the two 2-step clades 2-1 and 2-2 were nested, mainly distributed south and north of the CA-plain respectively.

The results of the addendum to the original NCPA proposed by Templeton (2001) to test for the occurrence of secondary contacts between previously isolated lineages are presented in Fig. 3, after excluding those populations lacking haplotype variation (samples 1-10, and 16-17), and therefore having average population clade distance equal to zero at all the nesting levels. For the three populations sampled in the nearby of the CA-plain (11, 13-14), where haplotypes from both clade 2-1 and 2-2 were found, the average population clade distance grew from the haplotype (0-step clades) to 2-step clade level, thus suggesting secondary contact as a likely explanation for the copresence in these populations of haplotypes from both the 2-step clades.

Population genetic structure and demographic analyses - The overall pattern of population differentiation was particularly marked and significant, with a F_{ST}=0.86 ($P < 0.01$). The F_{ST} estimates among population pairs (Table 3) showed a very wide variation, ranging from 0.00 to 1.00. These were also the only two values observed in pairwise comparisons between populations located north of the CS-plain (samples 1-10). Lower than average values of F_{ST} were observed among samples located within the two main calabrian plains (samples 11-13) and, particularly, among samples located south of CA-plain (samples 14-17). The differentiation between the samples located on alternative sides of the two Calabrian plains was instead very pronounced, the majority of pairwise F_{ST} values exceeding 0.60, with a few exceptions in the comparisons between samples located slighly north and south of the CA-plain (samples 11,13 vs. 14,15).

The AMOVA revealed that a large portion (63.65%) of the overall genetic variance observed in the mtDNA gene fragment analysed can be attributed to the difference between the three population groups, as defined by the two major phylogeographic discontinuities observed: north of CS-plain (samples 1-10), between the CS- and CA-plain (samples 11-13), south of CA-plain (samples 14-17). Nevertheless, a non-negligible part of the overall variation is due to differences among populations within groups (25.71%) and to differences within populations (10.64%), all variance components being highly significant ($P < 0.01$).

In order to ascertain a possible role for the geographic distances in shaping the observed patterns of genetic variation at mtDNA level, the association between genetic (F_{ST}) and geographic (in km) distance matrices was investigated at the level of both the entire dataset and by analysing separately the samples located on different sides of the CS-plain. A slight but significant correlation was observed between the two matrices at the level of the entire dataset ($r = 0.24$, $P < 0.05$; see Fig. 4). However, a stronger and significant association was observed when restricting the analysis to populations sampled south of CS-plain ($r = 0.60$, $P < 0.01$), whit no association being detected north of this area. Nevertheless, in no case was a significant correlation observed between residuals of the regressions and geographic distances.

The possibility of past demographic changes was investigated for each significant clade according to the NCPA, using the statistics F_S (Fu, 1997) and R_2 (Rasmos-Onsins & Rozas, 2002). The occurrence of a past population expansion was suggested only by the statistic R_2 (0.11; $P < 0.05$), and only for the clade 1-3, whereas no signs of past population growths were detected by the statistic F_S either for this clade or for any other clade.

5.3.2 ALLOZYMES

Genetic variability – 13 out of 21 loci analysed were monomorphic for the same allele in all the samples studied. The allele frequency at the polymorphic loci are presented in Table 4. No significant deviations from the expected Hardy-Weinberg equilibrium were detected across samples or loci.

At all loci that were polymorphic in more than one sample (all but *Mdh-1* and *Mpi*), alleles were found to be completely (*Mdh-2*^{115}, *Idh-2*^{107}, *Pgm-2*^{94}, *Gpi*^{94}) or almost completely (*Ldh-1*^{72}, *Pep-B*^{110}) confined to Calabria, south of CS-plain. Among these, the frequency of allele *Ldh-1*^{72} appeared to gradually decrease from south to north. When regressed over the geographic distance (in km) from the southernmost sample, the frequency of this allele appeared highly and significantly correlated with geography (Spearmann r = -0.81; $P < 0.05$). At the locus *Gpi*, high frequencies of the allele *Gpi*^{94} were observed in the two samples surveyed in central Calabria (11-12), whereas its frequency was never above 0.23 among samples located south of CA-plain.

Estimates of population genetic variability are given in Table 2. All the samples surveyed along the Apennines north of Calabria completely or almost completely lack genetic variation at all the parameters estimated. For instance, H_E observed among samples from this area was 0.02 (sample 9). By contrast, among samples from Calabria, H_E was never below 0.06, a value observed in the southernmost sample. The highest values of H_E were observed among the samples located slightly south of the CA-plain (14-16).

Population genetic structure and differentiation – The Neighbour-Joining analysis conducted on the pairwise Cavalli-Sforza & Edwards (1967) chord genetic distances (Fig. 5) showed that two main population clusters can be identified at allozyme level, comprising samples located alternatively north and south of the CS-plain. The same two population groups were also evident along the first principal component (explaining 74.6% of the total genetic variance), resulting from the PCA (see Fig. 6). According to this analysis, samples from the northern and central Apennines (1-9) formed a group of very close samples, with almost no identifiable intra-group variation, as expected from the allele frequency patterns (Table 4). Along the second principal component (explaining a further 19.5% of the total genetic variance), the samples located south of CS-plain are arranged roughly according to their geographic distribution, with the more geographically distant populations at the extremes and the populations from central Calabria being intermediate.

The overall pattern of population differentiation was found to be strong and highly significant, with an F_{ST} value among all populations of 0.47 ($P < 0.01$).

Pairwise estimates of F_{ST} are given in Table 3. A wide variation was found in pairwise comparison, with F_{ST} ranging from 0 to 0.76. Almost all comparisons among samples from the northern and central Apennines (samples 1-9) were very low and not significant, whereas F_{ST} values between these samples and those located in Calabria south of CS-plain were never below 0.32. With the single exception of comparisons involving the southernmost sample, pairwise comparisons among Calabrian samples located on the same side of the CA-plain always gave rise to $F_{ST} = 0$, whereas comparisons between samples located on different sides of the CA-plain ranged between 0.15 and 0.46.

The hierarchical AMOVA, based on the three groups defined for mtDNA data (i.e. according to their location with respect to the two main Calabrian plains), revealed that 52.44% of the overall allozyme genetic variance can be attributed to differences between groups, 43.85% to the within-population level of variation, and 3.71% to differences between populations within groups, all variance components being significant ($P < 0.01$).

As for mtDNA data, the relationship between genetic and geographic distances was investigated both at the level of the entire dataset, and by considering separately those samples located alternatively north and south of the CS-plain (see Fig. 4). At the level of the entire dataset a significant association was found between the two distance matrices ($r = 0.56$; $P < 0.01$). This association was, however, stronger when restricting the analysis only to the populations located south of CS-plain ($r = 0.85$; $P < 0.01$). By contrast, no significant association was found when analysing separately those populations surveyed north of CS-plain. In no case were significant relationships observed between the residuals of the regressions and geographic distances.

5.4 DISCUSSION

5.4.1 GENETIC DIVERSITY AND POPULATION STRUCTURE

In their anecdotal work, Nascetti *et al.* (1982) suggested a higher genetic diversity in populations of the Apennine yellow-bellied toad from Calabria compared to those from the northern portion of the species range, a pattern which was interpreted as evidence of a post-glacial northward range expansion from a refugial range in Calabria. Our analyses concerning both mitochondrial DNA and allozymes confirm this general pattern of population genetic diversity, giving further support to the proposed explanation and providing also several interesting data to gain further insight into the evolutionary processes that have shaped the present population genetic structure of *Bombina pachypus*.

Rapid range expansions are expected to produce a pattern of reduced genetic variation and population structure within the recently colonized areas because of serial founder events, particularly when long-distance colonization occurs (Hewitt, 1996 & 1999; Ibrahim *et al.*, 1996; Hewitt & Ibrahim, 2001). As a consequence, the pattern obtained when plotting pairwise genetic vs. geographic distances would reflect a lack or weakness of correlation between the two distance measures, and a degree of scattering which would be as much pronounced as stronger would be the habitat fragmentation within the newly colonized areas, and consequently the isolation among the newly established populations (Hutchinson & Templeton, 1999). Allozyme data indicated that among the populations north of Calabria there is a paucity of genetic differentiation, an almost complete lack of intrapopulation genetic diversity, an absence of correlation between pairwise genetic vs. geographic distances and a narrow degree of scattering. Following the above arguments, this genetic pattern appears fully consistent with a scenario of recent and rapid recolonization of the northern portion of the Italian peninsula by refugial populations in Calabria. This scenario is also supported by our mitochondrial data. In fact, range expansion was inferred from the NCPA as being the major process underlying the geographical distribution of clades nested within the clade 2-2, although it was not possible to establish from the NCPA whether it occurred through long-distance colonization possibly followed by subsequent fragmentation,

or by past fragmentation followed by range expansion. From the haplotype network topology, the haplotype h1 appears to be ancestral with respect to the other found north of CS-plain (e.g. Posada & Crandall, 2001). The presence of this haplotype fixed in 6 out of 10 populations, also comprising the southernmost populations sampled from this area (samples 9 and 10), as well as its wide distribution spanning the northern and central Apennines (see Fig.1 and Table 2), might suggest long-distance colonization as the most probable early event in the process of recolonization of northern environments. Furthermore, although long-distance dispersal is thought to be relatively uncommon in amphibians (but see Smith & Green, 2005 and references therein), the existence of rare long-distance dispersers was demonstrated for *B. variegata*, the sister species of the Apennine yellow-bellied toad (Szymura & Barton, 1991). The fact that the statistics F_S (Fu, 1997) and R_2 (Rasmos-Onsins & Rozas, 2002) failed to identify traces of a recent demographic expansion may be due to the very high degree of population subdivision detected at mitochondrial level among populations from this geographic area (Ray *et al.*, 2003). This strong population subdivision at the mtDNA (with F_{ST} worthing only 0.00 or 1.00) conflicts with the pattern of extensive genetic homogeneity observed at allozymes. This discrepancy could be explained by at least two mutually non-exclusive issues: *i)* a high degree of habitat fragmentation, coupled with the stronger genetic drift acting on the mtDNA with respect to nuclear markers (Birky *et al.*, 1989), or *ii)* a male-biased gene flow (Szymura *et al.*, 1985). The presence of distinct haplotypes fixed in three samples from the same hydrographic basin (samples 6, 7, and 8), while the same haplotype (h1) had been found fixed in very distant samples, leads us to favour causes acting on a local scale, as would be a strong genetic drift associated with habitat fragmentation, although a male-biased gene flow may also have contributed (see also Szymura *et al.*, 1985). Therefore, the substantial lack of genetic variation found in populations north of the CS-plain appears most likely due to both historical and contemporary factors, as also suggested by the NCPA (on this subject, see also the next section).

The only populations showing appreciable levels of genetic diversity were found in Calabria south of the CS-plain, with particularly high values of genetic diversity at both mtDNA and allozymes in populations from central Calabria.

Furthermore, all the analyses concur in showing that the genetic variation in these populations has a strong geographical component, and a north-south intergradation (see Figg. 4 and 6). Distinguishing between primary and secondary intergradation can be difficult, particularly when extensive intermixing has occurred and there is weak palaeogeographic evidence. However, in our case study, both genetic and historical information suggest the occurrence in Calabria of an allopatric differentiation, with the CA-plain acting as a barrier to dispersal, followed by a secondary contact. At the mtDNA, the geographic distribution of haplotypes (Table 2 and Fig.1), the pattern of population differentiation (Table 3) and the NCPA (Figg. 1 and 2), concordantly suggested the CA-plain as the source of a major phylogeographic break. Furthermore, NCPA indicated a long-distance colonization of southern Calabria possibly coupled with subsequent fragmentation, or a past fragmentation followed by range expansion, as the most likely historical events explaining the observed pattern. However, samples located slightly south of CA-plain are also among the most variable of the entire dataset, which is in striking contrast with the reduction of genetic variation expected if they had arisen through a long-distance colonization (see above). This genetic pattern does, however, appear consistent with a history of past fragmentation at the level of the CA-plain, followed by a secondary contact, an inference also strengthened by the addendum to the NCPA proposed by Templeton (2001; see Fig. 3). At allozyme level, clinal variation along a north-south axis was observed at the single locus *Ldh-1*, whereas at the locus *Gpi* a significant variation of the allele frequencies was observed among samples located on different sides of the CA-plain. Simulation studies indicated that primary intergradation does not produce clinal variations at neutral loci, whereas it is yielded by secondary contacts between previously differentiated lineages (Nichols & Hewitt, 1994; Durrett *et al.*, 2000). However, the absence of a clear straightforward linear transect (see also Santucci *et al.*, 1996), and the small number of samples from Calabria do not allow us to perform classical statistical cline analyses; inferences from single-locus clines are, moreover, hardly reliable, particularly in relation to the neutral/non-neutral nature of the cline (e.g. Endler, 1977; Kruuk *et al.*, 1999). A closer sampling in southern Italy and the application of new marker loci (microsatellites) are therefore in progress, and could help clarify this issue. A further

line of evidence in favour of a secondary contact comes from the comparisons of genetic vs. geographic distances. At both mtDNA and allozymes, a significant pattern of isolation-by-distance was detected among Calabrian samples, although it was more pronounced in the latter (R^2=0.719) than the former (R^2=0.362). Nevertheless, the lack of increased variance among differentiation estimates with increasing geographical distance allows us to reject the migration-drift equilibrium under the Hutchinson & Templeton (1999) criteria. The observed population genetic structure in Calabria appears therefore attributable to an historical intermixing between previously differentiated groups, rather than to a current balance between migration and drift. In this frame, the weak signs of past fragmentation at allozymes is thus indicative of the extensive intermixing that occurred between the two lineages.

From the overall genetic pattern reported it seems that two geographic areas, the CS- and CA- plains, have played a major role as historical barriers to dispersal, with the latter also giving rise to an event of allopatric differentiation. The present knowledge of palaeogeographic history in southern Italy strongly supports this hypothesis (Ghisetti, 1978 & 1981; Tortorici, 1981; Caloi *et al.*, 1989), depicting the CA- and CS- plains as two of the main grabens in southern Italy. During the Pleistocene they underwent alternate rising and sinking movements and were repeatedly marine-flooded as a consequence of the glacio-eusthatic sea level fluctuations, which render environmental conditions unfavourable to the dispersal of terrestrial species along the north-south axis until the late Pleistocene (Caloi *et al.*, 1989). Central and northern Calabria were connected during the early Pleistocene by a narrow land bridge (the emerging Catena Costiera), owing to the submersion of the CS-plain. The gradual process of erosion and deposition from the adjacent lands led the two plains to finally emerge during the middle-late and late Pleistocene. In the case of the Apennine yellow-bellied toad, it is plausible that unfavourable environmental conditions persisted during the early phases after the plains' emergence, as suggested by the present distribution of the species found at mid-altitude areas along the Apennine chain (e.g. Caputo *et al.*, 1985; Doria & Salvidio 1994; Mazzotti *et al.*, 1999; Sarrocco & Bologna, 2000) but not in the main Italian lowlands. If we apply the clock calibration of 1.3-1.6% per million years proposed

by Garcìa-Parìs & Jockusch (1999) for cytochrome *b* of Discoglossid frogs to the 0.6% of divergence among *Bombina* lineages, the coalescence time would be dated at 375,000-462,000 years. Although we are aware of the inaccuracies of the molecular clocks (e.g. Ayala, 1997 & 1999; Gibbons, 1998; Penny, 2005), these estimates would suggest that the key events in the history of populations of *B. pachypus* have occurred roughly since the Middle Pleistocene. This is in agreement with the palogeographic scenario so far described. Calabria south of CS-plain has been identified as the refuge for *B. pachypus* during glacial stages. The recolonization of northern habitats following climatic amelioration and the crossing of the present CA-plain would have been prevented by both marine transgressions and subsequent sub-optimal environmental conditions. In the late Pleistocene, conditions favourable to dispersal through the two plains would have been re-established, in agreement with the paleogeographical data and coalescence time. It is likely that the crossing of the CA-plain, and thus the secondary contact between the differentiated lineages, occurred at that time (i.e. at the approaching of the last ice age, after the Eemian transgression). Most probably, the recolonization of northern habitats would have occurred later, at the end of this period (the Würm).

Interestingly, growing evidence confirms the main role of the two Calabrian plains in shaping the patterns of distribution of species and evolutionary lineages within them. From classical biogeography it is well established that several taxa found in Calabria south of CA-plain have greater affinities with Sicilian taxa than with those from central and northern Calabria, and that the two plains represent the southern or northern edge of the range of many species (e.g. Audisio; 1984; Pignatti, 1984; Caloi *et al.*, 1989). More recently, genetic investigations are providing further evidence that these plains have repeatedly acted as barriers to dispersal (Santucci *et al.*, 1996 and maybe Podnar *et al.*, 2005). These findings therefore suggest that the two plains, and particularly the CA-plain, could be sites of suture zones (Remington, 1968), although further investigations focused on other taxa will be necessary in order to strengthen this hypothesis.

At first glance, the genetic pattern we observed within *B. pachypus* could be regarded as a typical pattern of "southern richness and northern purity" (Hewitt, 1999). However, our results also indicated that this southern richness is at least in

part due to allopatric differentiation within the southern glacial refugium followed by intermixing of previously differentiated lineages, and that a prolonged demographic stability of southern populations (e.g. Hewitt, 1996 & 2000) cannot explain the observed pattern of genetic diversity by itself, although neither can it be completely ruled out. Most probably, both these processes have played a role (see also Gomez & Lunt, 2004). Furthermore, the paleogeographic history of southern Italy and a growing body of data also suggest that this scenario could be more influential than was previously thought. These findings therefore indicate that, as previously proposed for the Iberian peninsula (Sanz *et al.*, 2000; Guillame *et al.*, 2000; see Gomez & Lunt, 2004 for a review of evidence), allopatric differentiation may have played an important role in shaping genetic diversity also within the southern Italian refugium, thus suggesting that this pattern could be a common feature of refugial ranges and stimulating further investigations on this matter both for this and other putative refugia. Finally, a point that deserves particular attention is the necessity for an appropriate sampling scheme so that this pattern and its strength may be properly appreciated (see also Taberlet, 1998; Gomez & Lunt, 2004). In fact, several case studies showing higher genetic variability in populations from southern Italy have been carried out with only one or very few samples from that region (e.g. Ragghianti & Wake, 1986; Capula & Ceccarelli, 2003), or with samples only from northern Calabria and Sicily, but not central and southern Calabria (e.g. Randi *et al.*, 2003; Fritz *et al.*, 2005). With such sampling schemes, it is difficult to disentangle the complex evolutionary dynamics that could have shaped genetic diversity within this southern refugium.

5.4.2 CONSERVATION IMPLICATIONS

As mentioned above, the conservation status of *B. pachypus* has recently been a cause for concern. The main threats to this species include: *i)* habitat loss and fragmentation, with consequent population isolation, *ii)* small and probably declining population size, and *iii)* the recent finding of chytridiomycosis in samples from the northern Apennines (e.g. Caputo *et al.*, 1985; Doria & Salvidio, 1994 and references therein; Sarrocco & Bologna, 2000; Stagni *et al.*, 2002). The genetic data

we have presented here add further cause for concern, but also provides useful information for species management strategies.

The importance of genetic diversity for the long-term persistence of populations, species and (more recently) ecosystem functions, has long been acknoledged (e.g. Young & Clarke, 2000; Frankham *et al.*, 2002; Frankham, 2005). Our results unequivocally indicate Calabria south of the CS-plain as the major reservoir of genetic diversity for *Bombina pachypus*. This geographic area acted as a refugium during the ice ages, and has been place of various evolutionary processes that are among the most influential in the history of this species, such as past fragmentation, range expansion and secondary contact. Therefore, protecting this genetic diversity reservoir is of the greatest importance for the conservation of the species. Unfortunately, although a large portion of the region is devoted to protected areas, the effectiveness of this protection is in many cases doubtful. For instance, the southernmost population we sampled (sample 17) is located on the southern edge of the Aspromonte National Park, but the imminent activation of a dam will soon erase the sampling site and its surroundings. Moreover, within the Serre National Park (which is a little south of the CA-plain) several breeding sites recently disappeared because of activities related to tree-cutting and water-draining practices (Bagnoli C. pers. comm.).

All populations sampled north of Calabria appeared completely (at the mtDNA) or almost completely (at allozymes) lacking in intrapopulation genetic diversity (see Table 2). This reduction of genetic diversity compared to the Calabrian samples is of clear historical origin, northern habitats having recently been recolonized from the south. Nevertheless, the analysis of the pattern of population differentiation at mtDNA and the NCPA suggested a further contribution by a current reduction of gene flow/dispersal. This pattern of present fragmentation and isolation is supported by the finding of three private haplotypes, each one fixed in a single population (haplotypes h5, h3 and h4 from samples 6, 7 and 8 respectively) from the Volturno river basin. After population subdivision, the achievement of reciprocal monophyly at mtDNA may occur quickly: ~4*Ne* generations, where *Ne* is the (equal) effective size of the two population fragments (Neigel & Avise, 1986). Although as far as we know there are no available estimates for the Apennine

yellow-bellied toad, the *Ne* in amphibians has been generally estimated as under a hundred individuals (Jehle & Arntzen, 2002; Beebee & Griffiths, 2005). It may thus be argued that the observed pattern of population subdivision could be of very recent, anthropogenic origin. This hypothesis is also plausible when considering that the underlying geographic area has been heavily affected by human activities in recent decades. The inconsistency of intrapopulation genetic diversity in the central and south-central peninsula, and the observed pattern of population subdivision at mtDNA, both suggest the need of an accurate planning of conservation practices for the Apennine yellow-bellied toad. These ought to include: *i)* an accurate assessment of the patterns of population connectivity and its enhancement where necessary, *ii)* the long-term monitoring of trends in population demography and fitness-related traits and, where also suggested by this latter investigation, *iii)* the restoration of population genetic variation. This latter management practice has the potential to dramatically reverse negative population trends, as has been shown by studies in both natural and laboratory reared populations (e.g. Madsen *et al.*, 1999; Hedrick & Kalinowski, 2000). However, before the appropriate source population can be indicated, fitness-related phenotypic differences ought to be properly evaluated.

5.5 TABLES AND FIGURES

Table 1. Geographic location and sample size for the 17 populations sampled of *Bombina pachypus* .

	Sampling locality	Elevation (m a.s.l.)	Geographical coordinates		Sample size	
			Latitude (N)	Longitude (E)	mtDNA	allozymes
1	Bagno di Romagna	465	43°51’	11°57’	13	14
2	Rio a Buti	200	43°56’	11°6’	11	11
3	Firenze	100	43°47’	11°14’	8	8
4	Rocca Caramanico	1050	42°09’	14°1’	6	6
5	Stipes	880	42°15’	12°58’	6	5
6	San Biagio	400	41°37’	13°54’	5	-
7	San Lupo	330	41°16’	14°39’	11	11
8	Carife	700	41°01’	15°12’	4	-
9	S Angelo a Fasanella	280	40°27’	15°20’	9	13
10	S. Severino Lucano	870	40°05’	16°4’	8	-
11	Monte Cocuzzo	1200	39°14’	16°10’	9	11
12	Macchia Longa	1300	39°22’	16°36’	12	12
13	Taverna	520	39°01’	16°35’	5	-
14	Cardinale	560	38°44’	16°24’	5	5
15	Girifalco	300	38°49’	16°29’	10	15
16	Stilo	300	38°30’	16°25’	11	11
17	Gambarie	1300	38°10’	15°50’	15	16

Table 2. Genetic variation in the 17 populations sampled of the Apennine yellow-bellied toad.

Sample	mtDNA			Allozymes			
	Haplotype [n]	*h*	$\pi(\cdot 10^2)$	A	*P*	H_O	H_E
1	h2 [13]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.00	0.00 (0.00)	0.00 (0.00)
2	h1 [11]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.00	0.00 (0.00)	0.00 (0.000
3	h1 [8]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.05	0.01 (0.01)	0.01 (0.01)
4	h1 [6]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.05	0.02 (0.02)	0.01 (0.01)
5	h1 [6]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.00	0.00 (0.00)	0.00 (0.00)
6	h5 [5]	0.000 (0.000)	0.000 (0.000)	-	-	-	-
7	h3 [11]	0.000 (0.000)	0.000 (0.000)	1.0 (0.0	0.05	0.01 (0.01)	0.01 (0.01)
8	h4 [4]	0.000 (0.000)	0.000 (0.000)	-	-	-	-
9	h1 [9]	0.000 (0.000)	0.000 (0.000)	1.1 (0.1	0.09	0.02 (0.01)	0.02 (0.01)
10	h1 [8]	0.000 (0.000)	0.000 (0.000)	-	-	-	-
11	h6 [7], h10 [1],	0.417 (0.191)	0.074 (0.019)	1.2 (0.1	0.19	0.08 (0.03)	0.08 (0.03)
12	h7 [8], h6 [3], h10 [1]	0.530 (0.136)	0.190 (0.049)	1.2 (0.1	0.23	0.09 (0.04)	0.09 (0.04)
13	h8 [2], h6 [1], h9 [1],	0.900 (0.161)	0.234 (0.053)	-	-	-	-
14	h11 [3], h12[1], h9 [1]	0.700 (0.218)	0.201 (0.082)	1.3 (0.1	0.29	0.12 (0.05)	0.10 (0.04)
15	h11 [9], h13 [1]	0.2000 (0.1541)	0.100 (0.077)	1.3 (0.1	0.33	0.11 (0.04)	0.11 (0.04)
16	h11 [11]	0.000 (0.000)	0.000 (0.000)	1.3 (0.1	0.29	0.10 (0.04)	0.10 (0.04)
17	h11 [15]	0.000 (0.000)	0.000 (0.000)	1.3 (0.1	0.29	0.06 (0.02)	0.06 (0.02)

h haplotype diversity; π nucleotide diversity; *A* average number of alleles per locus; *P* proportion of polymorphic loci; H_O observed heterozigosity; H_E expected heterozigosity (Nei's 1978 unbiased estimate).

Table 3. Pairwise estimates of F_{ST} for the 13 samples of *Bombina pachypu*s for which both mitochondrial and allozymes data were collected (samples are numbered as in Table 1). Allozymes estimates (according to Weir & Cockerham, 1984) are above the diagonal; mitochondrial estimates (incorporating Tamura & Nei, 1993 genetic distances) are below the diagonal.

Samples	1	2	3	4	5	7	9	11	12	14	15	16	17
1	-	0.00	0.04	0.19	0.00	0.12	0.06*	0.76*	0.67*	0.52*	0.54*	0.46*	0.74*
2	1.00*	-	0.02	0.18	0.00	0.10	0.01	0.70*	0.59*	0.39*	0.46*	0.36*	0.71*
3	1.00*	0.00	-	0.05	0.00	0.06	0.00	0.66*	0.54*	0.32*	0.42*	0.32*	0.66*
4	1.00*	0.00	0.00	-	0.08	0.11	0.06*	0.62*	0.52*	0.32*	0.41*	0.33*	0.65*
5	1.00*	0.00	0.00	0.00	-	0.04	0.00	0.65*	0.54*	0.34*	0.41*	0.33*	0.66*
7	1.00*	1.00*	1.00*	1.00*	1.00*	-	0.00	0.69*	0.59*	0.40*	0.45*	0.36*	0.66*
9	1.00*	0.00	0.00	0.00	0.00	1.00*	-	0.67*	0.57*	0.35*	0.43*	0.34*	0.64*
11	0.92*	0.84*	0.81	0.78*	0.78*	0.91*	0.82*	-	0.00	0.25*	0.25*	0.26*	0.46*
12	0.84*	0.76*	0.72	0.70*	0.70*	0.83*	0.74*	0.49*	-	0.15*	0.16*	0.15*	0.39*
14	0.90*	0.83*	0.79	0.75*	0.75*	0.89*	0.81*	0.40*	0.56*	-	0.00	0.00	0.29*
15	0.92*	0.88*	0.86	0.84*	0.84*	0.91*	0.86*	0.60*	0.67*	0.00	-	0.00	0.19*
16	1.00*	1.00*	1.00	1.00*	1.00*	1.00*	1.00*	0.80*	0.76*	0.17	0.01	-	0.19*
17	1.00*	1.00*	1.00	1.00*	1.00*	1.00*	1.00*	0.83*	0.79*	0.24	0.04	0.00	-

*$P < 0.05$ after 1000 permutations. Values ≤ 0 were in all cases set to 0.

Table 4. Allele frequencies at the 8 polymorphic loci detected among 13 populations of *Bombina pachypus* (populations numbered as in Table 1)

	Populations												
Locus/allele	1	2	3	4	5	7	9	11	12	14	15	16	17
Ldh-1													
72	----	----	----	----	----	0.14	0.12	0.27	0.35	0.20	0.39	0.45	0.94
100	1.00	1.00	1.00	1.00	1.00	0.86	0.88	0.73	0.65	0.80	0.61	0.55	0.06
Mdh-1													
100	1.00	1.00	1.00	1.00	1.00	1.00	0.92	1.00	1.00	1.00	1.00	1.00	1.00
110	----	----	----	----	----	----	0.08	----	----	----	----	----	----
Mdh-2													
100	1.00	1.00	0.94	1.00	1.00	1.00	1.00	0.95	1.00	0.90	0.93	0.95	0.89
112	----	----	0.06	----	----	----	----	----	----	----	----	----	----
115	----	----	----	----	----	----	----	0.05	----	0.10	0.07	0.05	0.11
Idh-2													
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.68	0.55	0.70	0.53	0.59	0.84
107	----	----	----	----	----	----	----	0.32	0.45	0.30	0.47	0.41	0.16
Pep-B													
100	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.13	0.28	0.37	0.17	0.40	0.07
110	----	----	----	----	----	----	0.04	0.87	0.72	0.63	0.83	0.60	0.93
Pgm-2													
94	----	----	----	----	----	----	----	0.23	0.10	0.10	0.17	0.04	0.09
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.77	0.90	0.90	0.83	0.96	0.91
Mpi													
96	----	----	----	----	----	----	----	----	----	----	0.07	----	----
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93	1.00	1.00
Gpi													
94	----	----	----	----	----	----	----	1.00	0.85	0.20	0.17	0.23	0.17
100	1.00	1.00	1.00	0.83	1.00	1.00	1.00	----	0.15	0.80	0.83	0.77	0.83
110	----	----	----	0.17	----	----	----	----	----	----	----	----	----

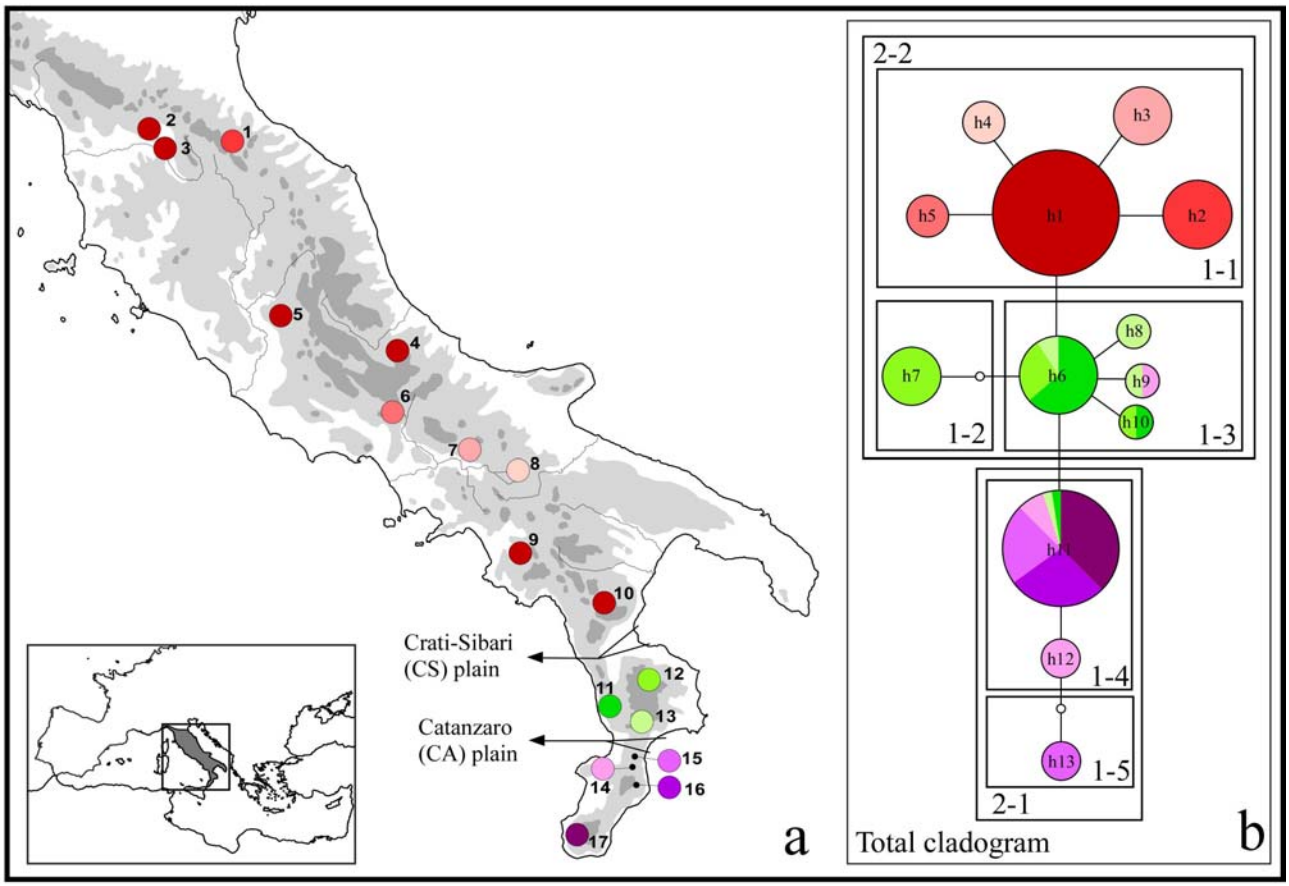

Figure 1. (a) Geographic range of *Bombina pachypus* in Italy and geographic location of the 17 population sampled. Localities are numbered as in Table 1. Altitudes are indicated by gray shadings (light grey indicates 200 to 1000 m, dark grey indicates above 1000 m). (b) Minimum spanning network showing relationships among the 13 haplotypes observed among the 17 population sampled. Haplotypes are presented as pie-diagrams, whose dimension is proportional to the number of times each haplotype was observed, and slices are proportional to its distribution among the sampled populations (which are numbered as in Table 1). Open circles are missing intermediate. Boxes represent the nesting design resulting from the application of rules in Templeton *et al.* (1987).

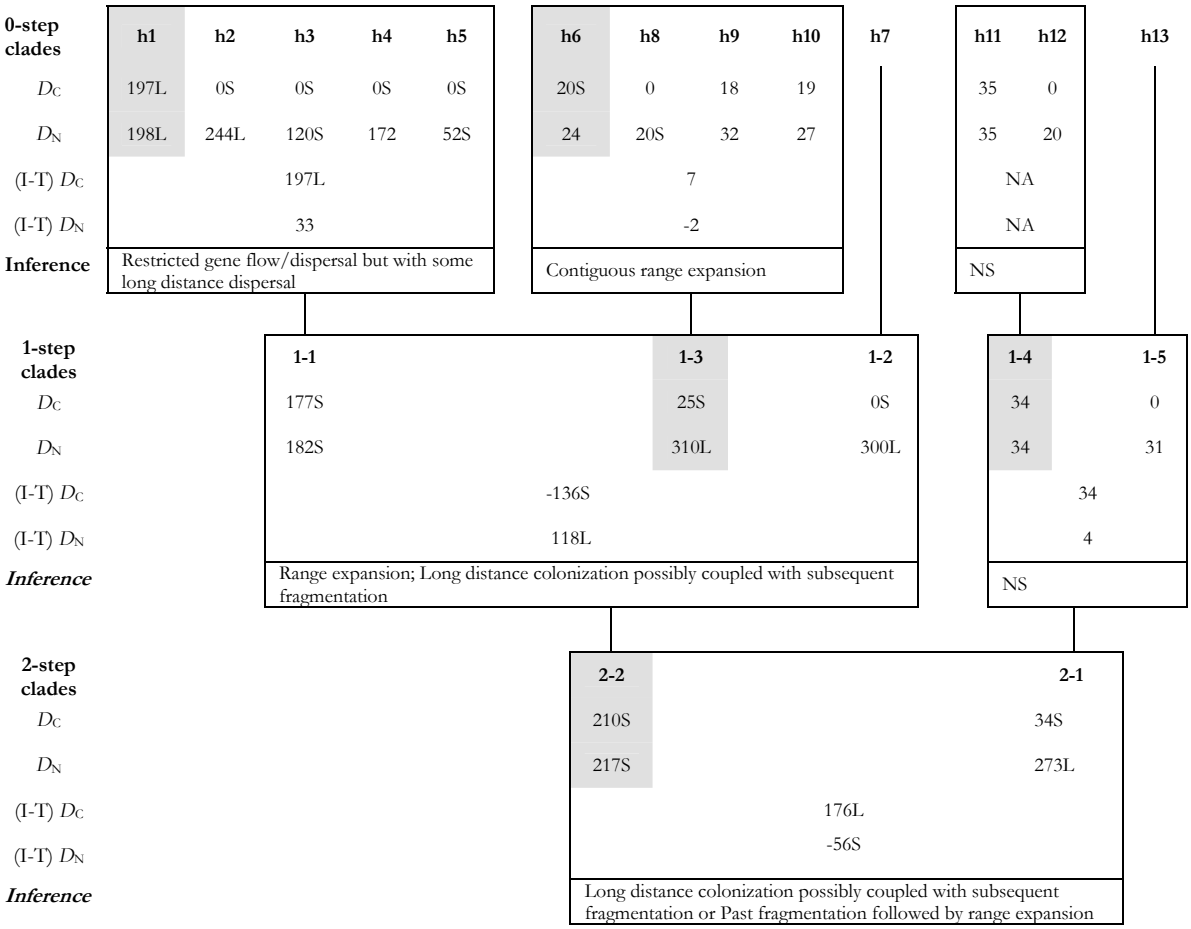

Figure 2. Nested clade phylogeographical analysis for the geographical distribution of the mitochondrial cytochrome *b* haplotypes in *Bombina pachypus*. D_C and D_N are the clade and nested clade distance measures (in km). I-T indicates interiors vs. tips contrasts for D_C and D_N measures of the corresponding clade. L and S indicate significantly larger or smaller than expected distance values. Interior clades and the associate distance measures are grey-shaded. The phylogeographic inferences resulting from the application of Templeton's (2004) inference key are also presented, for each clade showing a significant geographical association as detected by the nested contingency analysis.

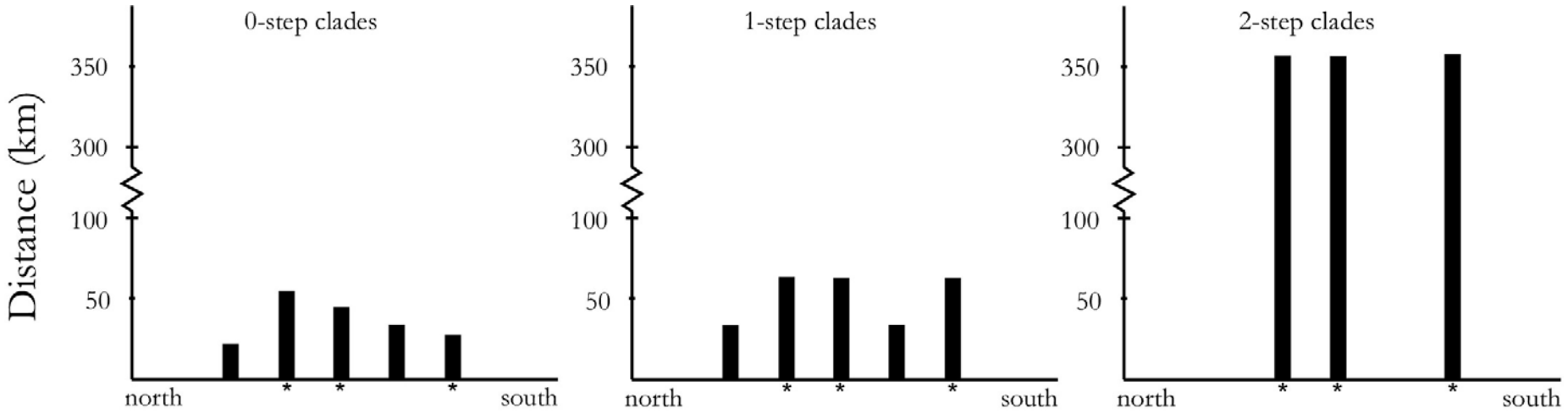

Figure 3. Average pairwise population distances for haplotypes and clades for the *Bombina pachypus* cladogram. Only populations showing haplotype variation are presented, and their average distances for each nesting level are plotted in order of decreasing latitude. * Samples where haplotypes from both the 2-step clades were found.

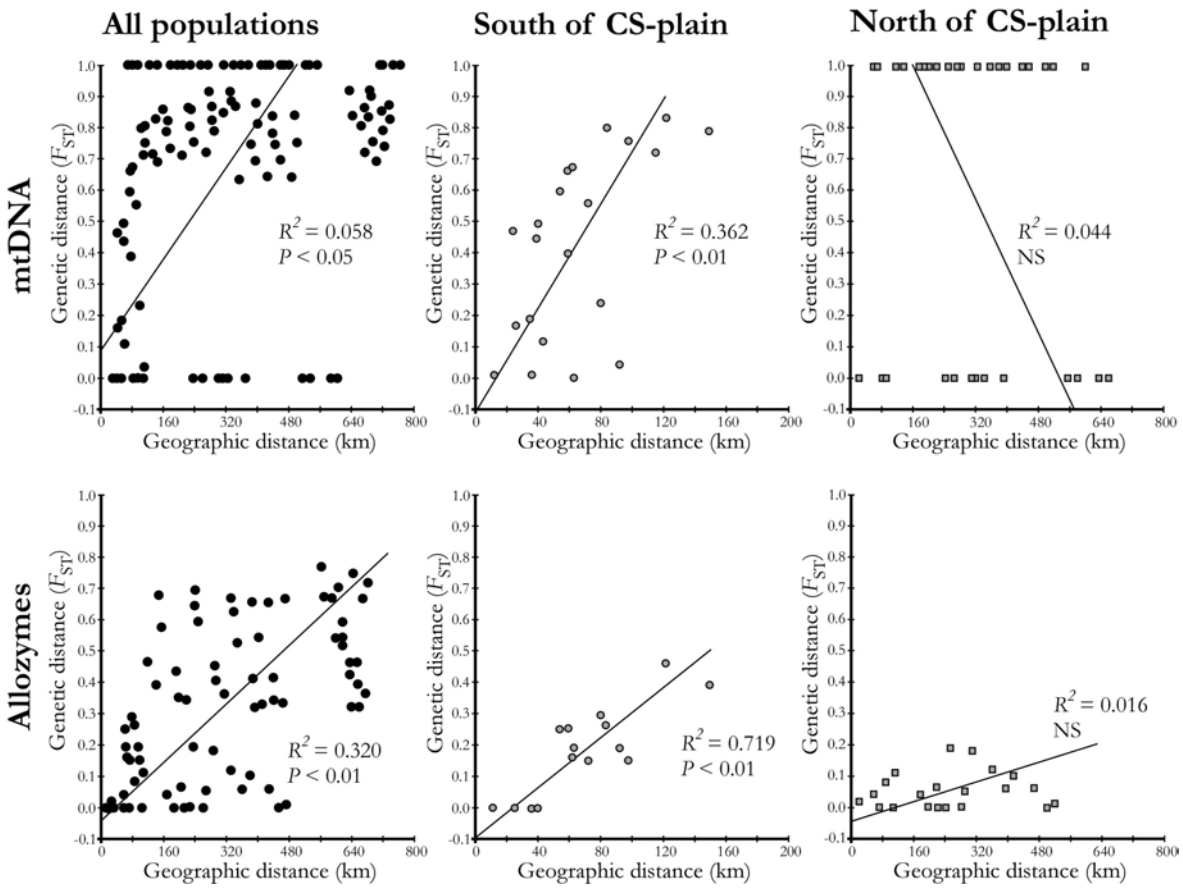

Figure 4. Relationships among genetic (F_{ST}) and geographic (in km) distances for population pairs of *Bombina pachypus*, carried out on both mtDNA and allozyme data. The analyses were performed at the level of the entire dataset as well as considering samples located north and south of CS-plain separately.

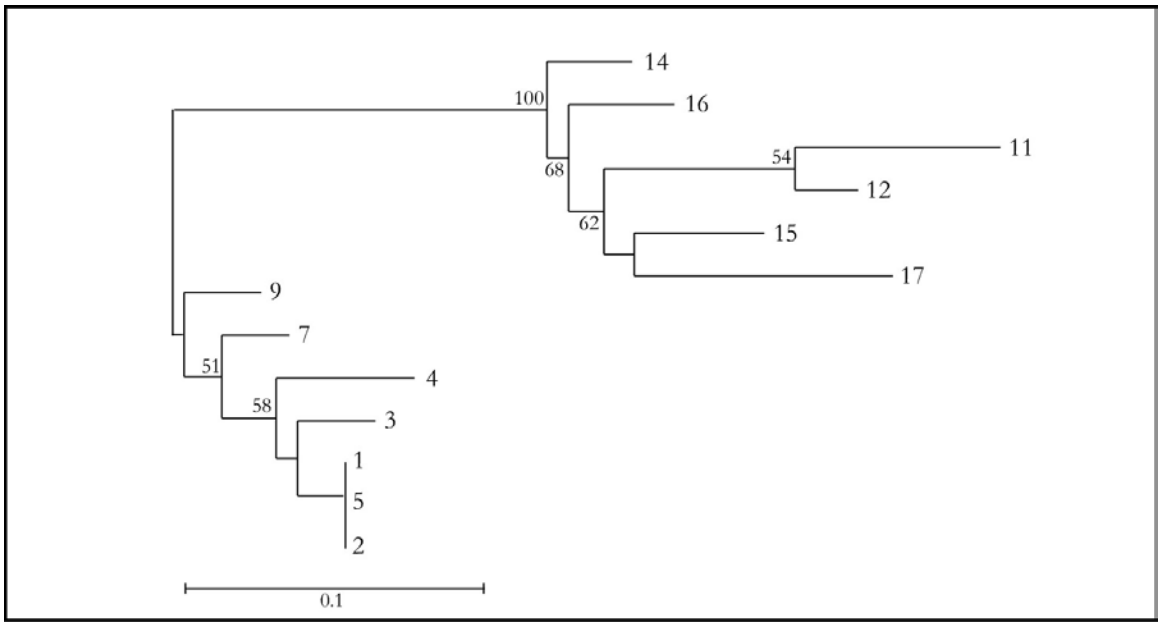

Figure 5. Neighbour Joining phenogram of the 13 populations of *Bombina pachypus* studied at allozymes, based on the pairwise Cavalli-Sforza & Edwards (1967) chord distances. Boostrap values > 50% after 1000 pseudoreplicates are shown. Samples are numbered as in Table 1.

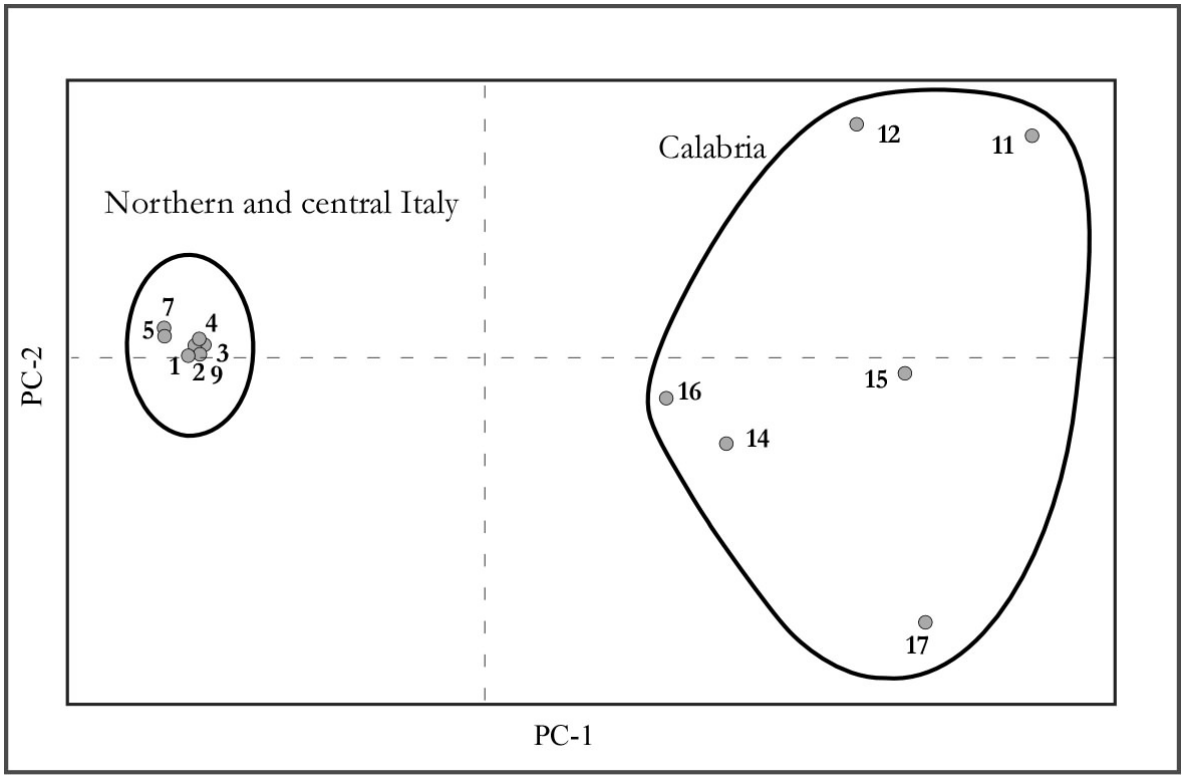

Figure 6. Principal component analysis of allele frequencies among the studied samples of *Bombina pachypus*. Both the axes were significant ($P < 0.01$) over 10,000 randomizations. Samples are encoded as in Table 1.

6. DISCUSSIONE GENERALE

6.1 FILOGEOGRAFIA COMPARATA

Come precedentemente ricordato, ormai da molti anni lo studio della diversità genetica di numerose specie europee, sia animali che vegetali, ha messo in evidenza l'importante ruolo svolto dalle penisole mediterranee come aree rifugio durante le culminazioni glaciali del Quaternario. L'ipotesi maggiormente accreditata (Hewitt, 1996, 1999 & 2000), vede queste penisole come aree dove le popolazioni sarebbero state caratterizzate da una prolungata stabilità demografica, fatto che in molti casi avrebbe anche consentito il mantenimento di una maggiore variabilità genetica rispetto alle popolazioni più settentrionali, che da esse si sarebbero originate mediante un processo di ricolonizzazione implicante ripetuti colli di bottiglia. Il pattern di distribuzione della diversità genetica risultante è stato quindi definito di "southern richness, northern purity" (Hewitt, 1996, 1999 & 2000). Recentemente, in studi di filogeografia per taxa della penisola iberica, alcuni autori (Sanz *et al.*, 2000; Guillame *et al.*, 2000; per una rassegna v.d. Gomez & Lunt, 2004) hanno suggerito l'ipotesi che i pattern di diversità genetica sovente osservati all'interno di questo rifugio possano in realtà farsi derivare da fenomeni di differenziamento allopatrico, eventualmente seguiti da contatto secondario, verificatisi all'interno della penisola stessa. Prescindendo dalle dinamiche coinvolte nella successiva fase di ricolonizzazione, sia della porzione settentrionale della penisola stessa sia dell'Europa (in proposito si vedano gli importanti lavori di Bilton *et al.*, 1998 e Petit *et al.*, 2003), per organismi come gli anfibi (si veda paragrafo 1.1) le due ipotesi sopra esposte offrono due modelli predittivi assai diversi circa i pattern di diversità genetica all'interno delle penisole meridionali. Nel primo caso, essendo la prolungata stabilità demografica l'elemento caratterizzante le popolazioni all'interno dei rifugi (modello P.D.S.), l'attesa è di un sostanziale equilibrio a livello regionale tra migrazione e deriva, un differenziamento modesto tra le popolazioni riconducibile per lo più ad isolamento per distanza, l'assenza di importanti

discontinuità filogeografiche e conseguentemente l'esistenza di un certo grado di concordanza filogeografica anche tra specie ecologicamente diverse. Nel secondo caso, il processo evolutivo maggiormente influente nel determinare i pattern di diversità sarebbe il differenziamento allopatrico all'interno della penisola stessa (modello R.W.R.), perciò l'attesa sarebbe di un elevato differenziamento genetico tra le popolazioni, la presenza di importanti discontinuità nella distribuzione geografica della variazione genetica ed un grado di concordanza filogeografica difficilmente prevedibile, ma con tutta probabilità basso tra specie ecologicamente diverse. Primo obiettivo di questa tesi, lo ricordiamo, era quello di valutare quale sia stato il contributo relativo dei due scenari P.D.S. e R.F.R. nel determinare i patterns di diversità genetica in taxa della penisola italiana. Ebbene, lo studio della variazione genetica e della sua distribuzione geografica nella raganella italiana, la salamandrina dagli occhiali e l'ululone appenninico, indica chiaramente che in queste specie il modello maggiormente esplicativo dei pattern di diversità osservati è quello R.W.R. Infatti, in tutte le specie studiate sono state individuate importanti discontinuità filogeografiche ed un forte grado di differenziamento genetico tra le popolazioni, rivelando come la componente geografica della variazione sia certamente quella di maggior rilievo. Inoltre, sia la localizzazione geografica delle discontinuità, sia i pattern di differenziamento, sia la probabile datazione dei principali eventi storici implicati, sono risultati complessivamente diversi nelle specie studiate. Tale forte discordanza filogeografica, se da una parte era attesa in virtù della strategia di campionamento di specie adottata, dall'altro suggerisce fortemente che il modello R.W.R. possa avere una valenza più generale, in particolare per la fauna anfibia.

Quattro aree geografiche sono state individuate complessivamente come sedi di discontinuità nella distribuzione della variazione genetica. Di seguito, per ciascuna di esse verranno ricordati i principali eventi storici che le hanno caratterizzate e che sembrano aver lasciato traccia nella struttura genetica delle specie studiate, insieme ad una breve rassegna delle evidenze di letteratura circa il loro ruolo come barriere alla dispersione, con particolare riguardo agli anfibi.

La più settentrionale delle discontinuità è stata osservata in Appennino settentrionale. In prossimità di quest'area è stata trovata infatti la zona di intergradazione tra la forma settentrionale e quella meridionale di raganella italiana.

Qui risiede inoltre il limite settentrionale dell'areale sia della salamandrina dagli occhiali sia dell'ululone apenninico, nonchè di diversi altri taxa dell'Italia peninsulare, tra i quali diversi elementi della fauna ittica (per una rassegna si veda Bianco, 1994) e tra gli anfibi la *Rana italica*, la *Salamandra s. gigliolii* e la forma peninsulare e siciliana di *Rana lessonae*. Per quanto riguarda quest'ultima specie in particolare è interessante notare come, sebbene studi di genetica biochimica abbiano evidenziato una variazione genetica geograficamente concorde con quella da noi riscontrata nella raganella italiana, il pattern di divergenza e la storia evolutiva delle popolazioni siano apparsi del tutto diversi. Per questa specie infatti, la forma padana è risultata più affine alle popolazioni centro europee che non a quelle dell'Italia peninsulare (Uzzell, 1979; Uzzell & Hotz, 1979; Snell *et al*., 2005), fatto che ha indotto gli autori ad ipotizzare per l'entità europea e padana l'esistenza di un secondo rifugio, o in Italia settentrionale (di indefinita collocazione) o in Ungheria ad est dei Carpazi (presso un'area già identificata come rifugio per diverse altre entità sia animali che vegetali; v.d. Snell *et al*., 2005e riferimenti all'interno). A ridosso dell'Appennino settentrionale ha sede la zona di transizione tra l'ecoregione padana e quella definita italiana a foreste semi-decidue e a sclerofille (EEA, 1998), e l'area costituisce inoltre lo spartiacque tra due distinti bacini di drenaggio. È quindi facile comprendere come mai qui risieda il limite della distribuzione geografica di molte forme. Inoltre, durante le culminazioni glaciali in questo tratto della catena appenninica erano presenti ghiaccai sparsi, ed in generale è possibile supporre che le condizioni climatiche risultassero fortemente sfavorevoli per l'attraversamento da parte di taxa termofili (Cattani, 2003; Cremaschi, 2003a & 2003b).

Una seconda discontinuità è stata evidenziata in Campania centrale, in corrispondenza del bacino di drenaggio del fiume Volturno. A nord e sud di quest'area si estendono gli areali di distribuzione rispettivamente di *Salamandrina perspicillata* e *S. terdigitata*, le due distinte specie di salamandrina dagli occhiali individuate come tali in questo studio. Nel capitolo 3 è stato suggerito un possibile scenario evolutivo per la divergenza tra queste due entità. In base alla ricostruzione offerta, la piana del Volturno non sarebbe all'origine della discontinuità osservata. Tuttavia la distribuzione dei campioni e della variazione genetica sembrano suggerire che in tempi più recenti quest'area possa aver svolto un ruolo come barriera alla

dispersione. Le ricostruzioni geo-morfologiche e paleoambientali supportano questa ipotesi (Romano *et al.*, 1994; Barra *et al.*, 1996 e riferimenti all'interno). Infatti dati biostratigrafici ottenuti da pozzi profondi indicano che già all'inizio del Quaternario vigevano in quest'area condizioni tali da determinare sedimentazione marina. Durante il Pleistocene l'area è stata interessata da subsidenza (in media 2-3 mm l'anno) compensata però da sedimentazione, condizioni che dettero luogo ad una alternanza di ambienti variabili tra il marino poco profondo e il subaereo (Barra *et al.*, 1996). In particolare, nella seconda metà del Pleistocene superiore, una diminuzione della subsidenza e la concomitante oscillazione glacio-eustatica del livello marino avrebbero consentito l'emersione totale dell'area e lo sviluppo di una importante fase di erosione subaerea (Romano *et al.*, 1994). É dunque verosimile che durante le fasi in cui l'area era sostanzialmente marina, essa abbia costituito una efficace barriera alla dispersione delle salamandrine lungo l'asse nord-sud. In proposito, vale la pena di menzionare che in quest'area risiede anche la zona di parapatria tra le due specie di ofidi *Zanemis longissimus* e *Z. lineatus*, recentemente riconosciute come distinte specie (Lenk & Wüster, 1999), e che dati preliminari sulla variazione mitocondriale in *Salamandra s. gigliolii* (Canestrelli & Nascetti, dati non pubblicati) suggeriscono che anche per essa quest'area sia stata efficace nel limitare la dispersione, in questo caso tra due linee evolutive pienamente cospecifiche. Sebbene non per quanto riguarda la fauna anfibia, anche nel caso di quest'area vi sono specie che vedono qui il confine del loro areale. Tra queste ricordiamo *Glossiphonia complanata*, *Erpobdella testacea* (Hirudinea; Minelli, 1977); *Dryops vienensis*, *D. striatopunctatus*, *D. sulcipennis* (Coleoptera, Dryopidae; Olmi, 1978); *Cneta marsicana*, *Boophthora erythrocephala* (Diptera, Simuliidae; Rivosecchi 1978); *Aplexa hypnorum*, *Lymnaea stagnalis*, *Anisus vortex* (Gasteropoda, Pulmonata; Girod *et al.*, 1980).

Infine, le discontinutà filogeografiche più meridionali sono state osservate presso le piane calabresi di Crati-Sibari e Catanzaro. Come ricordato nel capitolo 5, anch'esse avrebbero esercitato la loro influenza come barriere alla dispersione principalmente grazie all'interazione tra eventi geologici ed oscillazioni glacioeustatiche del livello del mare. Tali eventi hanno profondamente influenzato la storia di numerose specie, sia animali che vegetali, che vedono ad una di queste due aree il confine del loro areale (v.d. cap. 5). La piana di Catanzaro sembra essere stata

particolarmente influente, come suggerisce la maggior affinità di molti taxa della Calabria meridionale con entità della Sicilia, piuttosto che non con entità della Calabria settentrionale (v.d. cap. 5). La storia recente delle popolazioni di ululone appenninico è risultata sostanzialmente legata alle vicende paleogeografiche di queste due aree. Ai fini dell'obiettivo generale del presente lavoro, è interessante qui notare che il pattern di variazione geografica della diversità genetica da noi osservato per *Bombina pachypus* in Calabria, trova ampi parallelismi con quello individuato in *Rana lessonae* (Santucci *et al.*, 1996), *Talpa romana* (Ungaro *et al.*, 2001) e *Podarcis sicula* (Podnar *et al.*, 2005), che sono anche le uniche tre specie animali la cui variazione genetica in quest'area geografica sia stata investigata con un dettaglio di campionamento sufficiente ad evidenziare un pattern riconducibile a differenziamento allopatrico seguito da contatto secondario. Il quadro complessivo che si viene a delineare suggerisce dunque che le due piane, e particolarmente la piana di Catanzaro, potrebbero essere sedi di zone di sutura, un'ipotesi che per la sua rilevanza sia evoluzionistica che conservazionistica andrà di certo ulteriormente indagata in futuro. Ciò suggerisce la necessità di riconsiderare, ed eventualmente integrare in futuro, i risultati e le conseguenti inferenze ottenute attraverso schemi di campionamento inappropriati ad evidenziare tale pattern. Diversi studi precedenti infatti sono stati condotti campionando in Sicilia, Calabria a nord della piana di Crati-Sibari ed Appennino centro settentrionale, ma non in Calabria centrale e/o meridionale (ad es. Randi *et al.*, 2003; Fritz *et al.*, 2005), mentre diversi altri lavori si basano su un singolo o pochi campioni da quest'area (ad es. Ragghianti & Wake, 1986; Capula & Ceccarelli, 2003). Alla luce dei risultati ottenuti, è possibile affermare che studi effettuati con simili schemi di campionamento potrebbero aver portato ad una sottostima del ruolo di fenomeni micro-evolutivi (differenziamento allopatrico e contatto secondario) nel determinare i pattern di diversità genetica lungo la penisola italiana, in modo particolare nei casi in cui la maggior variabilità dei campioni meridionali è stata considerata un'indicazione della prolungata stabilità demografica delle popolazioni.

In merito a quanto fin qui discusso, particolarmente in relazione alle considerazioni di biogeografia storica, i risultati da noi presentati suggeriscono vigorosamente la necessità di una nota di cautela. Infatti, gran parte della

biogeografia storica si basa sull'analisi dei pattern di distribuzione delle specie, e la concordanza di tali pattern in taxa diversi viene usata per fare inferenze circa la loro possibile comune storia evolutiva (per quanto riguarda gli anfibi si veda ad es. Duellman 1999 ed i numerosi riferimenti bibliografici all'interno). Tra l'altro, la supposta relazione tra comune pattern di distribuzione geografica e comune storia evolutiva sta alla base della frequente costruzione di cladogrammi di area multi-specie, pratica talvolta utilizzata anche in studi di filogeografia comparata (ad es. Taberlet *et al.*, 1998). L'esistenza o meno di questa relazione è di grande importanza anche ai fini di una questione a lungo dibattuta, ossia l'origine delle attuali comunità biotiche. Secondo un'ipotesi classica (ad e.s. Mengel, 1964; citato in Sullivan *et al.*, 2000) intere comunità di organismi avrebbero risposto in modo concertato alle vicende paleoclimatiche. Una crescente mole di dati, in particolare relativa alla porzione settentrionale della fascia Oloartica (ad e.s. Bennett, 1997; FAUNMAP, 1996; Hewitt, 1999) supporta però l'ipotesi alternativa, che vede invece le comunità come entità largamente mutevoli, per effetto della risposta indipendente e diversificata delle specie ai medesimi eventi storici. Discernere tra queste due ipotesi ha almeno due importanti implicazioni. Da un lato, se l'ipotesi della risposta indipendente avesse una valenza generale, ciò comporterebbe che i fenomeni di coevoluzione si sarebbero verosimilmente instaurati in tempi molto recenti, visto il carattere effimero e l'origine recente delle comunità (Sullivan *et al.*, 2000; Hewitt, 2004b). Inoltre, fatto di estrema rilevanza, le sopracitate analisi biogeografiche sarebbero prive di significato, perchè equivarrebbero alla ricerca di correlazioni tra dati casuali (Sullivan *et al.*, 2000). I risultati da noi ottenuti, in particolare con l'ululone appenninico e la salamandrina dagli occhiali, indicano chiaramente che per queste specie tale relazione è ben lungi dal sussistere. Le "due" specie, pur condividendo quasi in pieno la distribuzione geografica nonché quella altitudinale, hanno avuto storie evolutive completamente diverse, tanto che in una (*B. pachypus*) la divergenza genetica osservata tra popolazioni ai margini opposti dell'areale è risultata tutto sommato modesta, mentre nell'altra i livelli ed i pattern di divergenza sono risultati tali da indicare persino l'esistenza di due distinte specie, di probabile divergenza tardo-miocenica. Inoltre, dati preliminari (Canestrelli & Nascetti, dati non publicati) relativi alla variazione genetica in altri due anfibi ampiamente

codistribuiti con i precedenti sia dal punto di vista latitudinale che altitudinale, *Salamandra s. gigliolii* e *Rana italica*, confermano quanto appena detto rispetto all'ululone appenninico e alla salamandrina dagli occhiali, indicando un forte differenziamento geografico delle popolazioni, correlabile in parte con gli eventi paleoclimatici precedentemente ricordati, cui le due specie hanno però risposto in modo diverso e completamente indipendente. Ancora, come ricordato poco sopra l'Appennino settentrionale rappresenta la principale discontinuità filogeografica sia nella raganella italiana che nella rana verde. Le forme peninsulari di queste specie hanno dunque distribuzione geografica sovrapposta, ma come poc'anzi illustrato la loro storia evolutiva è stata profondamente diversa. L'importanza di quanto appena detto risulterà più chiara ricordando che, mentre per ciò che riguarda l'Europa settentrionale e centrale esiste un generale accordo circa l'origine recente dell'attuale comunità biotica (Bilton *et al.*, 1998; Stewart & Lister, 2001; Petit *et al.*, 2003; Hewitt, 2004b), in riferimento alle aree mediterranee meridionali l'ipotesi della risposta concertata e di un origine tutto sommato "antica" delle comunità sembrerebbero ricevere in realtà supporto sia dai modelli che vedono queste come aree di stabilità demografica e come sorgenti per le successive ricolonizzazioni degli habitat settentrionali (ad es. Hewitt, 1996; Petit *et al.*, 2003), sia di quelli che ne enfatizzano il ruolo come sedi di elevata endemicità, a causa dell'effetto barriera svolto dalle principali catene montuose durante il processo di espansione degli areali verso nord (ad es. Bilton *et al.*, 1998). Invece, i dati qui presentati accreditano anche per queste aree il modello della risposta indipendente e dell'origine recente, suggeriscono la necessità di una maggiore cautela nel valutare le possibili implicazioni eco-evolutive dei pattern di distribuzione geografica, ed in ciò costituiscono un esplicito invito a valutare in futuro la generalizzabilità dei risultati stessi anche rispetto ad altri taxa della penisola italiana.

6.2 IMPLICAZIONI PER LA CONSERVAZIONE

I risultati presentati nei capitoli precedenti hanno importanti implicazioni per la conservazione sia delle singole specie studiate sia della batracofauna italiana in generale. Infatti, se da un lato l'esistenza delle importanti discontinuità filogeografiche poc'anzi ricordate andrà considerata in fase di pianificazione degli interventi di conservazione delle specie qui studiate, dall'altro si dovrà tenere conto della localizzazione geografica di tali discontinuità anche nella programmazione delle misure di gestione di quelle entità, in particolare della fauna anfibia, per le quali mancano ad oggi studi specifici circa i pattern di distribuzione della diversità genetica. Tuttavia, va enfatizzato che il carattere assolutamente prioritario di tali studi in materia di conservazione emerge con particolare chiarezza proprio sulla base dei risultati da noi ottenuti, in particolare con lo studio della variazione genetica in *Salamandrina terdigitata*. La salamandrina dagli occhiali è da più di cento anni il simbolo della zoologia italiana, per il fatto di essere l'unico genere di vertebrato terrestre endemico d'Italia, ed in ciò fornire all'Italia un "lustro" rispetto agli altri paesi europei (Lanza, 1988). Inoltre la perfetta concordanza tra l'areale di questa specie e quello di molte altre (v.d. sopra), l'assenza di vistose discontinuità nella distribuzione delle popolazioni lungo l'areale e l'apparente uniformità morfologica della specie, sono tutti elementi che fino ad oggi non avevano fatto presagire l'esistenza di alcun differenziamento significativo tra popolazioni. Il fatto che due distinte specie siano state individuate all'interno della salamandrina dagli occhiali, costituisce dunque per la fauna italiana il più sonoro monito fino ad oggi vibrato contro la pianificazione degli interventi di gestione delle specie in assenza di una accurata conoscenza della struttura e della distribuzione geografica della diversità genetica.

L'esistenza di una discrepanza tra marcatori mitocondriali e nucleari così profonda come quella osservata in *Hyla intermedia*, indica inoltre la necessità di diversificare la scelta dei marcatori, non solo ai fini di una più accurata comprensione di processi storico evolutivi, ma anche ai fini della genetica della conservazione, così da poter discernere dinamiche legate al singolo marcatore da quelle inerenti la specie nel complesso e le sue popolazioni. Ciò apparirà tanto più importante se si considera che, come già ricordato in precedenza, le specie di anfibi

qui studiate sono anche le uniche per la penisola italiana per le quali sono disponibili dati per entrambi i tipi di marcatori.

Infine, il fatto che la Calabria sia risultata la principale riserva di variabilità genetica in *Bombina pachypus*, *Salamandrina terdigitata* ed in diverse altre specie studiate in precedenza (ad es. Ragghianti & Wake, 1986; Santucci *et al.*, 1996; Ungaro *et al.*, 2001; Capula & Ceccarelli, 2003), unitamente al fatto che per alcune di esse quest'area sia stata teatro di vicende storico-evolutive particolarmente rilevanti (ed il numero di specie in cui ciò è avvenuto potrebbe essere stato ampiamente sottostimato, in virtù della strategia di campionamento sovente utilizzata; v.d. sopra), identifica quest'area geografica come quella probabilmente a maggiore priorità di conservazione di tutta l'Italia peninsulare.

RINGRAZIAMENTI

Ringrazio:

mia moglie Paola e mia figlia Alice, i miei genitori Cico e Carla, i miei fratelli Susanna, Stefano, Raffaella e Paolo, nonna Lina, Simone, Daniela ed i miei nipoti Michele, Davide, Jacopo e Giulia, perché esistono, danno senso alla mia vita e per aver scommesso su di me quando io non lo avrei fatto.

Il prof. Giuseppe Nascetti, per avermi offerto fin qui la possibilità di fare esattamente ciò che avrei voluto fare da grande, avermi fornito gli strumenti necessari per farlo, aver partecipato in spirito e corpo in particolare ad alcune delle fasi più dure e concitate di questo lavoro, e per avermi (...a modo suo) incoraggiato, spronato e mostrato fiducia in alcuni momenti di sconforto.

Tutti i miei compagni di lavoro, che a vario titolo e misura hanno messo a mia disposizione tempo, conoscenze e capacità, dando sempre pazientemente riscontro alle mie richieste: Pietro Bagalà, Claudio Bagnoli, Luciano Bullini, Roberta Cimmaruta, Vera Costantini, Veronica Della Rosa, Marcello Iaconelli, il povero Daniele Porretta (... con due erre), Alessandra Spanò, Sandra Urbanelli, Andrea Verardi e Francesca Zangari.

A tutti va la mia più profonda stima (e gratitudine) per l'elevatissimo grado di sopportazione dimostrato. A tutti auguro ogni bene.

BIBLIOGRAFIA GENERALE

Alford RA, Richards SJ (1999) Global amphibian declines: a problem in applied ecology. *Annual Review of Ecology and Systematics*, **30**, 133-165.

Amorosi A, Colalongo ML, Fusco F (1999) Glacio-eustatic control of continental shallow marine cyclicity from late Quaternary deposits of the southeastern Pò plain, northern Italy. *Quaternary Research*, **52**, 1-13.

Andreone F, Luiselli L (2000) The Italian batrachofauna and its conservation status: a statistical assessment. *Biological Conservation*, **96**, 197-208.

Arbogast B, Kenagy GJ (2001) Comparative phylogeography as an integrative approach to historical biogeography. *Journal of Biogoegraphy,* **28**, 819-825.

Arnaud-Haond S, Monteforte M, Blanc F, Bonhomme F (2003) Evidence for male-biased effective sex ratio and recent step-by-step colonization in the bivalve *Pinctada mazatlanica*. *Journal of Evolutionary Biology*, **16**, 790-796.

Audisio P (1984) I Kateretidae e i Nitidulidae (Coleoptera) dell'Italia meridionale: distribuzione ed ipotesi sul popolamento. *Biogeographia*, **10**, 605-623.

Austerlitz F, Mariette S, Machon N, Gouyon PH, Godelle B (2000) Effects of colonization processes on genetic diversity: differences between annual plants and tree species. *Genetics*, **154**, 1309-1321.

Austin JD, Lougheed SC, Neidrauer L, Chek AA, Boag PT (2002) Cryptic lineages in a small frog: The post-glacial history of the spring peeper, *Pseudacris crucifer* (Anura: Hylidae). *Molecular Phylogenetics and Evolution,* **25**, 316-329.

Avise JC (2004) *Molecular Markers, Natural History, and Evolution*, 2nd edn. Sinauer Associates, Sunderland, Massachusetts.

Avise JC (2000) *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.

Avise JC , Hamrick JL (1996) *Conservation Genetics: Case Histories from Nature*. Chapman & Hall, New York.

Avise JC, Aquadro CF (1982) A comparative summary of genetic distances in the vertebrates. *Evolutionary Biology*, **15**, 151-185.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics,* **18**, 489-522.

Ayala FJ (1997) Vagaries of the molecular clock. *Proceeding of the National Academy of Science, USA,* **94**, 7776-7783.

Ayala FJ (1999) Molecular clock mirages. *Bioassays*, **21**, 71-75.

Baer CF (1999) Among-locus variation in F_{ST}: Fish, allozymes and the Lewontin-Krakauer test revisited. *Genetics*, **152**, 653-659.

Baker CS, Megrano-Gonzalez L, Calambokidis J, Perry A, Pichler F, Rosenbaum H, Straley JM, Urban-Ramirez J, Yamaguchi M, Von-Ziegesar O (1998) Population structure of nuclear and mitochondrial DNA variation among humpback whales in the North Pacific. *Molecular Ecology*, **7**, 695–707.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729-744.

Barinaga M (1990) Where have all the froggies gone? *Science*, **247**, 1033-1034.

Barra D, Romano P, Santo A, Campajola L, Roca V, Tuniz C (1996) The versilian transgression in the Volturno River plain (Campania, Italy): palaeoenvironmental evolution and chronological data. *Il Quaternario*, **9**, 445-458.

Beebee TJ, 1996. *Ecology and conservation of amphibians*. Chapman & Hall, New York.

Beebee TJC, Griffiths RA (2005) The amphibian decline crisis: a watershed for conservation biology. *Biological Conservation*, **125**, 271-285.

Bennett KD (1997) *Evolution and Ecology, the Pace of Life*. Cambridge University Press, Cambridge, UK.

Bermingham E, Moritz C (1998) Comparative phylogeography: concepts and applications. *Molecular Ecology,* **7**, 367-369.

Bernatchez L, Wilson CC (1998) Comparative phylogeography of Neartic and Paleartic fishes. *Molecular Ecology,* **7**, 431-452.

Bianco PG (1994) L'ittiofauna continentale dell'Appennino umbro-marchigiano, barriera semipermeabile allo scambio di componenti primarie fra gli opposti versanti dell'Italia centrale. *Biogeographia*, **17**, 427-485.

Bilton DT, Mirol PM, Mascheretti S, Fredga K, Zima J, Searle JB (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 1219-1226.

Birky CWJ, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics*, **121**, 613-627.

Blaustein AR, Kiesecker JM (2002) Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, **5**, 597-608.

Blaustein AR, Johnson PTJ (2003) The complexity of deformed amphibians. *Frontiers in Ecology and the Environment*, **2**, 87-94.

Blaustein AR, Wake DB (1990) Declining amphibian populations: a global phenomenon? *Trends in Ecology and Evolution*, **5**, 203-204.

Blaustein AR, Wake DB (1995) The puzzle of declining amphibian populations. *Scientific American*, **272**, 52-57.

Bohonak AJ (2002) IBD (Isolation By Distance): A program for analyses of isolation by distance. *Journal of Heredity*, **93**, 153-154.

Borkin LJ (1999) Distribution of Amphibians in North Africa, Europe, Western Asia, and the Former Soviet Union. In: *Patterns of Distribution of Amphibians: a Global Perspective* (ed. Duellmann WE), pp 329-420. The Johns Hopkins University Press, Baltimore.

Brewer GJ, Sing C F (1970) *An introduction to Isozyme Techniques*. Academic Press, New York & London.

Broughton RE, Harrison RG (2003) Nuclear gene genealogies reveal historical, demographic, and selective factors associated with speciation in field crickets. *Genetics*, **163**, 1389-1401.

Brown WM, George MJr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceeding of the National Academy of Science, USA*, **76**,1967-1971.

Bruno S (1970) Anfibi e rettili di Sicilia. *Atti Accademia Gioenia di Scienze Naturali di Catania*, **7**, 1-144.

Bruno S (1973) Anfibi d'Italia: Caudata. *Natura*, **64**, 209-450.

Bruno S (1983) Lista rossa degli Anfibi italiani. *Rivista Piemontese di Storia Naturale*, **4**, 4-58.

Caccone A, Milinkovitch MC, Sbordoni V, Powell JR (1997) Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). *Systematic Biology*, **46**, 126-144.

Caloi L, Malatesta A, Palombo RM (1989) Biogeografia della Calabria meridionale durante il Quaternario. *Atti Accademia Peloritana dei Pericolanti classe I, Scienze Matematiche Fisiche e Naturali*, **67**, 261-278.

Canestrelli D (2002) Ricerche genetiche ed ecologiche su *Hyla italica*. MS.c. Thesis.

Capula M, Ceccarelli A (2003) Distribution on genetic variation and taxonomy of insular and mainland populations of the Italian wall lizard, *Podarcis sicula*. *Amphibia-Reptilia*, **24**, 483-495.

Caputo E, Kalbi M, De Filippo G (1985) Gli anfibi e i rettili del massiccio degli Alburni. *Natura*, **76**, 94-104.

Cattani L (2003) Considerazioni floristiche sull'evoluzione degli ambienti. In: *Italia preistorica* (eds Guidi A, Piperno M), pp 46-67. Edizioni La Terza, Italy.

Cavalli-Sforza LL, Edwards WF (1967) Phylogenetic analysis: models and estimation procedures. *Evolution*, **21**, 550-570.

Cheliak WM, Pitel JA (1984) *Techniques for starch gel electrophoresis of enzymes from forest tree species*. Information Report Pl-X-42. Petawawa National Forestry Institute. Canadian Forestry Service. Canada.

Cherty LM, Case SM, Wilson AC (1978) Frog perspective on the morphological difference between humans and chimpanzees. *Science*, **200**, 209-211.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657-1660.

Corsetti L, Angelini C (2000) *Salamandrina terdigitata*. In: *Anfibi e Rettili del Lazio* (eds Bologna MA, Capula M, Carpaneto GM), pp 38-39. Palombi editore, Italy.

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, **134**, 959-969.

Cremaschi M (2003a) Mutamenti del clima nel Quaternario: le linee generali. In: *Italia preistorica* (eds Guidi A , Piperno M), pp 3-15. Edizioni La Terza, Italy.

Cremaschi M (2003b) La penisola italiana nel Quaternario: aspetti geologici e geoarcheologici. In: *Italia preistorica* (eds Guidi A, Piperno M), pp 15-39. Edizioni La Terza, Italy.

Crochet PA, Chen JZ, Pons JM, Lebreton JD, Hebert PDN, Bonhomme F (2003) Genetic differentiation at nuclear and mitochondrial loci among large white-headed gulls: Sex biased interspecific gene flow? *Evolution*, **57**, 2865-2878.

Cucchiara V, Bologna MA (1996) Gli anfibi di sorgenti termali sulfuree nella provincia di Viterbo (p.3). Riassunti 1° Congresso Naz. S.H.I. (Torino 2 - 6 /10 /1996), 143 pp.

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. *Diversity and Distribution*, **9**, 141-150.

Daudin FM (1803) *Histoire Naturelle, Générale et Particulière des Reptiles*. vol. VIII. Paris: de l'Imprimerie de F. Dufart.

Deffontaine V, Libois R, Kotlik P *et al.* (2005) Beyond the Mediterranean peninsulas: evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (*Clethrionomys glareolus*). *Molecular Ecology*, **14**, 1727-1739.

Delfino M, Bailon S (2000) Early Pleistocene herpetofauna from Cava Dell'Erba and Cava Pirro (Apulia, Southern Italy). *Herpetological Journal*, **10**, 95-110.

Di Giovanni MV, Vlach MR, Giangiuliani G, Goretti E, Torricelli R (1998) Genetic analysis of the species of *Sigara s. str.* (Heteroptera, Corixidae) in the Italian Peninsula. *Italian Journal of Zoology*, **65**, 393-397.

Donnelly MA, Guyer C, Jutterbock JE, Alford RA (1994) Techniques for marking amphibians. In: *Measuring and monitoring biological diversity: standard methods for amphibians* (eds Heyer WR, Donnelly MA, McDiarmid RW, Hayek LC, Foster MS), pp 277-284. Smithsonian Books.

Doria G, Salvidio S (1994) *Atlante degli anfibi e rettili della Liguria*. Regione Liguria, Italy.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin*, **19**, 11-15.

Dubois A (1995) The valid scientific name of the Italian treefrog, with comments on the status of some early scientific names of Amphibia Anura, and on some articles of the Code concerning secondary homonyms. *Dumerilia*, **2**, 55-71.

Duellman WE (1993) *Amphibian Species of the World: Additions and Corrections*. Univ. of Kansas Printing Service. Lawrence, KS.

Duellmann WE (1999) *Patterns of Distribution of Amphibians: a Global Perspective*. The Johns Hopkins University Press, Baltimore.

Duermeijer CE, Van Vugt N, Langereis CG, Meulenkamp JE, Zachariasse WJ (1998) A major late Tortonian rotation phase of the Crotone Basin using AMS as tectonic tilt correction and timing of the opening of the Tyrrhenian Basin. *Tectonophysics*, **287**, 233-249.

Durrett R, Buttel L, Harrison R (2000) Spatial models for hybrid zones. *Heredity*, **84**,9-19.

EEA (1998) Digital map of European ecological regions. Available at: http://dataservice.eea.eu.int/dataservice/metadetails.asp?id=192.

Emerson BC, Hewitt GM (2005) Phylogeography. *Current Biology*, **15**:R367-371.

Endler JA (1977) *Geographic variation, speciation, and clines*. Princeton Univ. Press, Princeton, NJ.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479-491.

Faunmap (1996) Spatial response of mammals to late quaternary environmental fluctuations. *Science*, **272**, 1601-1606.

Felsenstein J (1993) Phylogeny inference package (*PHYLIP*), Version 3.5c. University of Washington, Seattle.

Ferrière R, Dieckmann U, Couvet D (2004) *Evolutionary Conservation Biology*. Cambridge University Press, Cambridge, UK.

Fitzinger LJ (1826) *Neue classification der Reptilien nach ihren natürlichen Verzeichnisse der Reptilien-Sammlung des K. Zoologischen Museums zu*. Wien.

Frankham R (2005) Ecosystem recovery enhanced by genotypic diversity. *Heredity*, **95**, 183.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK.

Fritz U, Fattizzo T, Guicking D, Tripepi S, Pennisi MG, Lenk P, Joger U, Wink M (2005) A new cryptic species of pond turtle from Southern Italy, the hottest spot in the range of the genus *Emys* (Reptilia, Testudines, Emydidae). *Zoologica Scripta*, **34**, 351-371.

Frost DR (2002) Amphibian species of the world: an online reference (V2.21). Electronic database available at http://research.amnh.org/herpetology/amphibia /index.html.

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**, 915-925.

Garcia-Paris M, Jockusch EL (1999) A mitochondrial DNA perspective on the evolution of Iberian *Discoglossus* (Amphibia: Anura). *Journal of Zoology*, **248**, 209-218.

García-París M, Alcobendas M, Buckley D, Wake DB (2003) Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. *Evolution*, **57**, 129-43.

Garcia-Paris M, Arano B, Herrero P (2001) Molecular characterisation of the contact zone between *Triturus pygmaeus* and *T. marmoratus* (Caudata: Salamandridae) in central Spain and their taxonomic assessment. *Revista Española de Herpetología*, **15**, 115-126.

Gardner T (2001) Declining amphibians population: a global phenomenon in conservation biology. *Animal Biodiversity and Conservation*, **24.2**, 25-44.

Gasc J-P, Cabela A, Crnobrnja-Isailovic J, Dolmen D, Grossenbacher K, Haffner P, Lescure J, Martens H, Martinez Rica JP, Maurin H, Oliveira ME, Sofianidou TS, Veith M, Zuiderwijk A (eds) (1997) *Atlas of Amphibians and Reptilesin Europe*. Societas Europaea Herpetologica & Muséum National d´Histoire Naturelle, Paris.

Ghisetti F (1979) Evoluzione neotettonica dei principali sistemi di faglie della Calabria centrale. *Bollettino della Società Geologica Italiana*, **98**, 387-430.

Ghisetti F (1981) L'evoluzione strutturale del bacino plio-pleistocenico di Reggio Calabria nel quadro geodinamico dell'arco calabro. *Bollettino Geologico Italiano*, **100**, 433-466.

Gibbons A (1998) Calibrating the mitochondrial DNA clock. *Science*, **279**, 38-39.

Girod A, Bianchi I, Mariani M (1980) *Gasteropodi 1 (Gastropoda: Pulmonata; Prosobranchia: Neritidae, Viviparidae, Bithyniidae, Valvatidae)*. Guide per il riconoscimento delle specie animali delle acque interne italiane. CNR, Italy.

Goebel AM, Donneley JM, Atz ME (1999) PCR primers and amplification methods for 12s Ribosomal DNA, the control region, cytochrome oxidase I and cytochrome b in bufonids, and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution*, **11**, 163-199.

Gómez A, Lunt DH (2004) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: *Phylogeography in Southern European Refugia: Evolutionary Perspectives on the origins and conservation of European Biodiversity* (eds Weiss S, Ferrand N). Kluwer Academic Publishers. Dordrecht, The Netherlands.

Goudet J (1999) PCAGEN, a computer package which performs Principal Component Analysis (PCA) on gene frequency data. Available at http://www2.unil.ch/izea/softwares/pcagen.html.

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

Goudet J, Perrin N, Waser P (2002) Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Molecular Ecology*, **11**, 1103-1114.

Greenwood PJ (1980) Mating systems, phylopatry and dispersal in birds and mammals. *Animal Behavior*, **28**, 1140-1162.

Guillaume GP, Heulin B, Arrayago MJ, Bea A, Brana F (2000) Refuge areas and suture zones in the Pyrenean and Cantabrian regions: geographic variation of the female MPI sex-linked alleles among oviparous populations of the lizard *Lacerta (Zootoca) vivipara*. *Ecography*, **23**, 3-10.

Haig SM (1998) Molecular contributions to conservation. *Ecology*, **79**, 413-425.

Hanken J (1999) Why are there so many new amphibian species when amphibians are declining? *Trends in Ecology and Evolution*, **14**, 7-8.

Hanski I (1994) A practical model of metapopulation dynamics. *Journal of Animal Ecology*, **63**, 151-162.

Hare MP (2001) Prospects for nuclear gene phylogeography. *Trends in Ecology and Evolution*, **16**, 700-706.

Harris H (1966) Enzyme polymorphism in man. *Proceeding of the Royal Society London Series B*, **164**, 298-310.

Harris H, Hopkinson DA (1976) *Handbook of enzyme electrophoresis in human genetics*. North-Holland Publishing Company Inc.

Hass CA, Dunski JF, Maxson LR (1995) Divergent lineages within the *Bufo margaritifera* complex (Amphibia: Anura; Bufonidae) revealed by albumin immunology. *Biotropica*, **27**, 238-249.

Hecnar SJ, Closkey M (1996) Regional dynamics and the status of amphibians. *Ecology*, **77**, 2091-2097.

Hedrick PW, Kalinowski ST (2000) Inbreeding depression in conservation biology. *Annual Review of Ecology and Systematics*, **31**, 139-162.

Helbig AJ, Salomon M, Bensch S, Seibold I (2001) Male-biased gene flow across an avian hybrid zone: evidence from mitochondrial and microsatellite DNA. *Journal Evolutionary Biology,* **14**, 277-287.

Hewitt GM (1996) Some genetic consequences of ice ages and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247-276.

Hewitt GM (1999) Postglacial re-colonization of european biota. *Biological Journal of the Linnean Society*, **68**, 87-112.

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907-913.

Hewitt GM (2004a) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions* of *the Royal Society of London. Series B, Biological Sciences*, **359**, 183 -195.

Hewitt GM (2004b) The structure of biodiversity - insights from molecular phylogeography. *Frontiers in Zoology*, **1**, 4.

Hewitt GM, Ibrahim KM (2001) Inferring glacial refugia and historical migrations with molecular phylogenies. In: *Integrating Ecology and Evolution in a Spatial Context.* BES Symposium volume, (eds Silvertown J, Antonovics J, Blackwells) pp271-294. Oxford.

Heyer WR, Donnelly MA, Mc Diarmid RW, Hayek L-A C, Foster MS (1994) *Measuring and monitoring biological diversity: standard methods for amphibians*. Smithsonian Institution Press, Washington, DC.

Hoelzer GA (1997) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees revisited. *Evolution*, **51**, 622-626.

Hutchinson DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution*, **53**, 1898-1914.

Ibrahim KM, Nichols RA, Hewitt GM (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity*, **77**, 282-291.

IUCN (2004) Global Amphibian Assessment. At: http://www.globalamphibians.org/.

Jaarola M, Searle JB (2004) A highly divergent mitochondrial DNA lineage of *Microtus agrestis* in southern Europe. *Heredity*, **92**, 228- 234.

Jehle R, Arntzen JW (2002) Microsatellite markers in amphibian conservation genetics. *Herpetological Journal*, **12**, 1-9.

Johns GC, Avise JC (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial Cytochrome-b gene. *Molecular Biology and Evolution*, **15**, 1481-1490.

Johnson JA, Toepfer JE, Dunn PO (2003) Contrasting patterns of mitochondrial and microsatellite population structure in fragmented populations of greater prairie-chickens. *Molecular Ecology,* **12**, 3335-3347.

Kawamura T, Nishioka M (1977) Reproductive biology of Japanese anurans. In: *Reproductive Biology* of *Amphibians* (eds Tylor DH, Guttman SI), pp 132. Plenum Press, New York.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequence. *Journal of Molecular Evolution*, **16**, 111-120.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceeding of the National Academy of Science, USA*, **86**, 6196-6200.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature*, **400**, 652-655.

Kruuk LEB, Baird SJE, Gale KS, Barton NH (1999) A comparison of multilocus clines maintained by environmental selection or by selection against hybrids. *Genetics*, **153**, 1959-1971.

Kumar SK, Tamura S, Jakobsen IB, Nei M (2001) MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics*, **17**, 1244-1245.

Lacépède BGE (1788) *Histoire naturelles des quadrupèdes ovipares et des serpens*. Paris, Imprimerie du Roi (Hôtel de Thou), vol. I.

Lampert KP, Rand AS, Mueller UG, Ryan MJ (2003) Fine-scale genetic pattern and evidence for sex-biased dispersal in the tungara frog, *Physalaemus pustulosus*. *Molecular Ecology*, **12**, 3325-3334.

Lanza B (1983a) *Anfibi e Rettili*. In: Guide per il riconoscimento delle specie animali delle acque interne italiane. CNR, Italy.

Lanza B (1983b) *Ipotesi sulle origini del popolamento erpetologico della Sardegna*. Lav. Soc. It. di Biogeogr. [new series], 8 (1980): 723-744.

Lanza B (1988) *Salamandrina terdigitata* (Lacépède, 1788): emblem of the Unione Zoologica Italiana. *Bollettino di Zoologia*, **55**, 1-4.

Lanza B, Canestrelli D (2002) Atypische Färbung bei *Salamandrina terdigitata* (Lacépède, 1788) und *Bufo viridis viridis* Laurenti, 1768. *Salamandra*, 38, 105-108.

Lanza B, Caputo V, Nascetti G, Bullini L (1995) *Morphologic and genetic studies of the European plethodontid salamanders: taxonomic inferences (genus* Hydromantes*)*. Monografie XVI, Museo Reg. Sc. Nat., Torino.

Lanza B, Corti C (1993) Erpetofauna italiana: acquisizioni ed estinzioni nel corso del novecento. *Supplemento Ricerche Biologia Selvaggina*, **21**, 5-49.

Lanza B, Nascetti G, Capula M, Bullini L (1984) Genetic relationships among west Mediterranean *Discoglossus* with the description of a new species (Amphibia Salientia Discoglossidae). *Monitore Zoologico Italiano*, **18**, 133-153.

Lanza B, Nascetti G, Capula M, Bullini L (1987) Les Discoglosses de la region mediterraneenne occidentale. *Bulletin de la Société Herpétologique de France*, **40**,16-27.

Lanza B, Vanni S (1991) Notes on the biogeography of the Mediterranean island amphibians. *Atti dei Convegni Lincei*, **85**, 335-344.

Ledig FT (1986) Heterozygosity, heterosis, and fitness in outbreeding plants. In: C*onservation Biology. The Science of Scarcity and Diversity*, (eds Soulé ME) pp 77-104. Sinauer Associates, Sunderland, Massachusetts.

Lee JE, Yang DE, Kim YR, Lee H, Lee HI, Yang SY, Lee, HY (1999) Genetic relationships of Korean treefrogs (Amphibia; Hylidae) based on mitochondrial cytochrome b and 12S rRNA genes. *Korean Journal Biological Science*, **3**, 295-301.

Lenk P, Wüster W (1999) A multivariate approach to the systematics of Italian rat snakes of the *Elaphe longissima* complex (Reptilia, Colubridae): revalidation of Camerano's *Callopeltis longissimus* var. *lineata*. *Herpetological Journal*, **9**, 153-162.

Lewontin RC, Krakauer J (1973) Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics*, **74**, 175-195.

Lips KR (1998) Decline of a tropical montane amphibian fauna. *Conservation Biology*, **12**, 106-117.

Macgregor HC, Sessions SK, Arntzen JW (1990) An integrative analysis of phylogenetic relationships among newts of the genus *Triturus* (family Salamandridae), using comparative biochemistry, cytogenetics and reproductive interactions. *Journal of Evolutionary Biology*, **3**, 329-373.

Madsen T, Shine R, Olsson M, Wittsell H (1999) Restoration of an inbred adder population. *Nature*, **402**, 34-35.

Malatesta A (1985) Cause e meccanismi delle variazioni di taglia e delle modificazioni strutturali nei mammiferi quaternari delle isole mediterranee. *Bollettino Società Paleontologica Italiana*, **24**, l95-199.

Marsh DM (2001) Fluctuations in amphibian populations: a meta-analysis. *Biological Conservation*, **101**, 327-335.

Marsh DM, Trenham PC (2000) Metapopulation dynamics and amphibian conservation. *Conservation Biology*, **15**, 40-49.

Martínez-Solano I, Gonçalves H, Arntzen JW, García-París M (2004) Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: *Alytes*). *Journal of Biogeography*, **31**, 603-618.

Maxson LR, Maxson RD (1979) Comparative albumin and biochemical evolution in Plethodontid salamanders. *Evolution*, **33**, 1057-1062.

Maynard Smith J , Haigh J (1974) The hitchhiking effect of a favorable gene. *Genetics Research,* **23**, 23-35.

Mazzotti S, Caramori G, Barbieri C (1999) Atlante degli Anfibi e dei Rettili dell'Emilia-Romagna (aggiornamento 1993/1997). *Quaderni della Stazione Ecologica del Museo Civico di Storia Naturale di Ferrara **12**, 1-121.*

Mengel RM (1964) The probable history of species formation in some northern wood warblers. *Living Bird*, **3**, 9-44.

Mertens R, Wermuth H (1960) *Die Amphibien und Reptilien Europas* (Dritte Liste, nach dem Stand vom 1. Januar 1960). Frankfurt/M. (Kramer).

Minelli A (1977) *Irudinei (Hirudinea).* Guide per il riconoscimento delle specie animali delle acque interne italiane. Roma, CNR.

Miola A, Albanese D, Valentini G, Corain L (2003) Pollen data for a biostratigraphy of LGM in the Venetian Pò plain. *Italian Journal of Quaternary Science*, **16**, 21-25.

Monsen KJ, Blouin MS (2003) Genetic structure in a montane ranid frog: restricted gene flow and nuclear-mitochondrial discordance. *Molecular Ecology*, **12**, 3275-3286.

Montuire S, Marcolini F (2002) Palaeoenvironmental significance of the mammalian faunas of Italy since the Pliocene. *Journal of Quaternary Science*, **17**, 87-96.

Moore WS (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nucelar-gene trees. *Evolution*, **49**, 718-726.

Moritz C, Faith DP (1998) Comparative phylogeography and the identification of genetically divergent areas for conservation. *Molecular Ecology*, **7**, 419-429.

Moritz C, Schneider CJ, Wake DB (1992) Evolutionary relationships within *Ensatina eschscholtzi* complex confirm the ring species interpretation. *Systematic Zoology*, **41**, 273-291.

Nascetti G, Androne F, Capula M, Bullini L (1988) A new *Salamandra* species from southwestern Alps (Amphibia, Urodela, Salamandridae). *Bollettino del Museo Regionale di Scienza Naturali di Torino* **6**, 617-638.

Nascetti G, Capula M, Lanza B, Bullini L (1985) Ricerche elettroforetiche su anfibi della regione mediterranea: aspetti tassonomici ed evolutivi. *Riassunti del IV congresso associazione Ghigi*, **45**.

Nascetti G, Cimmaruta R, Lanza B, Bullini L (1996) Molecular Taxonomy of European Plethodontid Salamanders (Genus *Hydromantes*). *Journal of Herpetology*, **30**, 161-183.

Nascetti G, Lanza B, Bullini L (1995) Genetic data support the specific status of the Italian treefrog (Amphibia: Anura: Hylidae). *Amphibia-Reptilia*, **16**, 215-227.

Nascetti G, Vanni S, Bullini L, Lanza B (1982) Variabilità e divergenza genetica in popolazioni italiane del genere *Bombina* (Amphibia, Discoglossidae). *Bollettino di Zoologia*, **49** (suppl.): 134-135.

Nei M (1972) Genetic distance between populations. *The American Naturalist*, **106**, 283-292.

Nei M (1975) *Molecular Population Genetics and Evolution*. North-Holland Publishing Company, Amsterdam.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **83**, 583-589.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Neigel JE (2002) Is Fst obsolete?. *Conservation Genetics*, **3**, 167-173.

Neigel JE, Avise JC (1986) Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In: *Evolutionary processes and theory* (eds Karlin S, Nevo E) pp 515-534. New York Academic Press.

Nevo E, Beiles A (1991) Genetic diversity and ecological heterogeneity in amphibian evolution. *Copeia*, **1991**, 565-592.

Nichols RA, Hewitt GM (1994) The genetic consequences of long distance dispersal during colonisation. *Heredity*, **72**, 312-317.

Olmi M (1978) *Driopidi, Elmintidi (Coleoptera Dryopidae, Elminthidae)*. Guide per il riconoscimento delle specie animali delle acque interne italiane. Roma, CNR.

Paillette M (1967) Valeur taxonomique des émissions sonores chez les *Hyla* (Amphibiens, Anoures) de la faune française. *Compte-rendus de l'académie des sciences, Paris, Séries D*, **264**, 1626-1628.

Palumbi SR, Baker CS (1994) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Bioogy. Evolutionary*, **11**, 426-435.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991) *The Simple Fool's Guide to PCR, Version 2.0*. Privately published document compiled by PALUMBI S., Dept. Zoology, Univ. Hawaii, Honolulu, HI, 96822.

Pechmann JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW (1991) Declining amphibian populations: the problem of separating human impacts from natural fluctuations. *Science*, **253**, 892-895.

Pechmann JHK, Wilbur HM (1994) Putting declining amphibian populations in perspective: natural fluctuations and human impacts. *Herpetologica*, **50**, 65-84.

Penny D (2005) Relativity for molecular clocks. *Nature*, **436**, 183-184.

Petit RJ, Aguinagalde I, de Beaulieu JL *et al.* (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. *Science*, **300**, 1563-1565.

Picariello O, Scillitani G, Cretella M (1990) Electrophoretic data supporting the specific rank of the frog *Rana graea italica* Dubois, 1985. *Amphibia-Reptilia*, **11**, 189-192.

Piel WH, Nutt KJ (2000) One species or several? Discordant patterns of geographic variation between allozymes and mtDNA sequences among spiders in the genus *Metepeira* (Araneae: Araneidae). *Molecular Phylogenetics and Evolution*, **15**, 414-418.

Pignatti S (1984) La flora dell'Appennino meridionale: distribuzione attuale ed ipotesi sull'origine. *Biogeographia*, **10**, 89-100.

Podnar M, Mayer W, Tvrtković N (2005) Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences. *Molecular Ecology*, **14**, 575-588.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Posada D, Crandall KA (2001) Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, ***16***, 37-45.

Posada D, Crandall KA, Templeton AR (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487-488.

Poulik MD (1957) Starch gel electrophoresis in a discontinuous system of buffers. *Nature*, **180**, 1477.

Ragghianti M, Wake DB (1986) Genic variation and its evolutionary implications in the Italian newt, *Triturus italicus*. *Herpetologica*, **42**, 206-214.

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, **19**, 2092-2100.

Randi E, Tabarroni C, Rimondi S, Lucchini V, Sfougaris A (2003) Phylogeography of the rock partridge (*Alectoris graeca*). *Molecular Ecology,* **12**, 2201- 2214.

Ravazzi C, Strick MR (1995) Vegetation change in a climatic cycle of Early Pleistocene age in the Leffe Basin (Northern Italy). *Palaeogeography, Palaeoclimatology, Palaeoecology*, **117**, 105-122.

Ray N, Currat M, Excoffier L (2003) Intra-deme molecular diversity in spatially expanding populations. *Molecular Biology and Evolution*, **20**, 76-86.

Remington CL (1968). Suture-zones of hybrid interaction between recently joined biotas. In: *Evolutionary biology* (eds Dobzhansky T, Hecht MK, Steere WC), pp 321–428. Plenum, New York.

Riberon A, Miaud C, Grossenbacher K, Taberlet P (2001) Phylogeography of the Alpine salamander, *Salamandra atra* (Salamandridae) and the influence of the Pleistocene climatic oscillations on population divergence. *Molecular Ecology,* **10**, 555-60.

Ribéron A, Sotiriou E, Andreone F, Miaud C, Taberlet P (2002) Lack of genetic diversity in *Salamandra lanzai* revealed by cytochrome b gene sequences. *Copeia*, **2002**, 229-232.

Rice, WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223-225.

Richards C M, Moore WS (1996) A phylogeny for the African treefrog family Hyperoliidae based on mitochondrial DNA. *Molecular Phylogenetics and Evolution*, **5**, 522-532.

Richardson BJ, Bayerstock PR, Adams M (1986) *Allozyme electrophoresis: a handbook for animal systematics and population studies.* Academic Press, San Diego, California, USA.

Ripplinger, JI, Wagner RS (2004) Phylogeography of northern populations of the pacific treefrog, *Pseudacris regilla. Northwestern Naturalist*, **85**, 118-125.

Rivosecchi (1978) *Simuliidi (Diptera, Simuliidae).* Guide per il riconoscimento delle specie animali delle acque interne italiane. CNR, Italy.

Roe BA, Ma DP, Wilson RK, Wong JFH (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J*ournal Biological Chemistry*, **260**, 9759- 9774.

Rogers JS (1972) Measures of genetic similarity and genetic distances. *Studies in Genetics,* **7213**,145-153.

Romano P, Santo A, Voltaggio M (1994) Evoluzione geomorfologica della piana del fiume Volturno (Campania) durante il tardo Quaternario (Pleistocene medio-superiore - Olocene). *Il Quaternario,* **7**, 41-56.

Rouchy JM, Orszag-Sperber F, Blanc-Valleron M-M, Pierre C, Riviere M, Combourieu-Nebout N, Panayedes I (2001) Paleoenvironmental changes at the Messinian-Pliocene boundary in the eastern Mediterranean: southern Cyprus basins. *Sedimentary Geology*, **145**, 93-117.

Rouchy JM, Pierre C, Et-Touhami M, Kerzazi K, Caruso A, Blanc-Valleron MM (2003) Late Messinian to Early Pliocene paleoenvironmental changes in the Melilla Basin (NE Morocco) and their relation to Mediterranean evolution. Sedimentary Geology, **163**, 1-27.

Rozas J, Sànchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496-2497.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Edition. Cold Spring Harbor Laboratory Press.

Santucci F, Nascetti G, Bullini L (1996) Hybrid zones between two genetically differentiated forms of the pond frog *Rana lessonae* in southern Italy. *Journal of Evolutionary Biology*, **9**, 429-450.

Sanz N, García-Marín JL, Pla C (2000) Divergence of brown trout (*Salmo trutta*) within glacial refugia. *Canadian Journal of Fisheries and Aquatic Sciences*, **57**, 2201-2210.

Sarrocco S, Bologna MA (2000) *Bombina variegata*. In: *Anfibi e Rettili del Lazio* (eds Bologna MA, Capula M, Carpaneto GM), pp 38-39. Palombi editore, Italy.

Savi P (1821) Descrizione (inedita) di una nuova specie di Salamandra terrestre, *Salamandra perspicillata* Nob. *Biblioteca italiana (Giornale di Letteratura, Scienze ed Arti)*, **22**, 228-230.

Schierup MH, Vekemans X, Charlesworth D (2000) The effect of subdivision on variation at multi-allelic loci under balancing selection. *Genet. Res. Camb.*, **76**, 51-62.

Schlotterer C (2003) Hitchhiking mapping - functional genomics from the population genetics perspective. *Trends in Genetics,* **19**, 32-38.

Schneider H (1974) Structure of mating calls and relationship of the European tree frogs (Hylidae, Anura). *Oecologia*, **14**, 99 - 110.

Schneider S, Roessli D, Excoffier L (1999) Arlequin ver 2.0: A software for population genetic data analysis. Univ. of Geneva Genet. Biometr. Lab: Geneva, Switzerland.

Schreiber E (1875) *Herpetologia Europaea*. Druck und Verlag von Friedrich Vieweg und Sohn, Braunschweig.

Scillitani G, Picariello O (2000) Genetic variation and its causes in the crested newt, *Triturus carnifex* (Laurenti, 1768), from Italy (Caudata: Salamandridae). *Herpetologica*, **56**, 119-130.

Scoccianti C (2001) *Amphibia: aspetti di ecologia della conservazione*. Editore Guido Persichino Grafica, Firenze, Italy.

Selander RK, Smith MH, Yang SY, Johnson WE, Gentry JB (1971) Biochemical polymorphism and systematics in the genus Peromyscus. I. Variation in the old-field mouse. In: *Studies in Genetics IV*, pp.49-90. University of Texas Publications, 7103.

Shaffer HB, Fisher RN, Davidson C (1998) The role of natural history collections in documenting species declines. *Trends in Ecology and Evolution*, **13**, 27-30.

Shaw KL (2002) Conflict between mitochondrial and nuclear DNA phylogenies of a recent species radiation: what mitochondrial reveals and conceals about modes of speciation in Hawaiian crickets. *Proceedings of the National Academy of Sciences, USA*, **99**, 16122-16127.

Skelly DK, Werner EE, Cortwright SA (1999) Long-term distributional dynamics of a Michigan amphibian assemblage. *Ecology*, **80**, 2326-2337.

Skelly DK, Yurewicz KL, Werner EE, Relyea RA (2003) Estimating decline and distributional change in amphibians. *Conservation Biology*, **17**, 744-751.

Smith MA, Green DM (2005) Are all amphibian populations metapopulations? Dispersal and the metapopulation paradigm in amphibian ecology and conservation. *Ecography* , **28**, 110-128.

Snell C, Tetteh J, Evans IH (2005) Phylogeography of the pool frog (*Rana lessonae*) in Europe: evidence for native status in Great Britain and for an unusual postglacial colonisation route. *Biological Journal of the Linnean Society*, **85**, 41-51.

Soltis DE, Haufler CH, Darrow DC , Gastony GJ (1983) Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *American Fern Journal* , **73**, 9-27.

Stagni G, Scoccianti C, Fusini R, (2002) Segnalazione di chytridiomicosi in popolazioni di *Bombina pachypus* (Anura, Bombinatoridae) dell'Appennino tosco-emiliano. IV° Congresso della *Societas Herpetologica Italica.* Ercolano, Italy.

Stallard RF (2001) Possible environmental factors underlying amphibian decline in eastern Puerto Rico: analysis of U.S. Government data archives. *Conservation Biology*, **15**, 943-953.

Stefani F, Galli P, Crosa G, Zaccara S, Calamari D (2004) Alpine and Apennine barriers determining the differentiation of the rudd (*Scardinius erythrophthalmus* L.) in the Italian peninsula. *Ecology of Freshwater Fishes*, **13**, 168-175.

Steinfartz S, Veith M, Tautz D (2000) Mitochondrial sequence analysis of Salamandra taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. *Molecular Ecology*, **9** , 397-410.

Stewart JR, Lister AM (2001) Cryptic northern refugia and the origins of the modern biota. *Trends in Ecology and Evolution*, **16**, 608-613.

Sullivan J, Arellano E, Rogers DS (2000) Comparative Phylogeography of Mesoamerican Highland Rodents: Concerted versus Independent Response to Past Climatic Fluctuations. *The American Naturalist*, **155**, 755-768.

Swofford DL (2003) PAUP*: Phylogenetic analysis using parsimony (* and other methods). Beta, Version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Swofford DL, Selander RB (1999) BIOSYS-2: A computer program for the analysis of allelic variation in population genetics and biochemical systematics (Release 2.0). University of Illinois, Urbana, Champaign, IL.

Szymura JM (1993) Analysis of hybrid zones with *Bombina*. In: *Hybrid zones and the evolutionary process*. (ed Harrison R), pp 261-289. Oxford University Press, Oxford.

Szymura JM, Barton NH (1991) The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*: comparisons between transects and between loci. *Evolution*, **45**, 237-261.

Szymura JM, Spolsky C, Uzzell T (1985) Concordant change in mitochondrial and nuclear genes in a hybrid zone between two frog species (genus *Bombina*). *Experientia*, **41**, 1469-1470.

Taberlet P (1998) Biodiversity at the intraspecific level: The comparative phylogeographic approach. *Journal of Biotechnology*, **64**, 91-100.

Taberlet P, Fumagalli L, Wust-Saucy A-G, Cosson J-F (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453-464.

Tajima F (1993) Simple methods for testing the molecular evolutionary clock hypothesis. *Genetics*, **135**, 599-607.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512-526.

Templeton AR (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**,779-791.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **13**, 789-809.

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with Haplotypes inferred from restriction endonuclease mapping and DNA sequence data. I. Basic theory and analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 342-351.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619-633.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767-782.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659-669.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876-4882.

Ting TC, Tsaur SC, Wu CI (2000) The phylogeny of closely related species as revealed by the genealogy of a speciation gene, Odysseus. *Proc. Natl. Ac. Sc., USA*, **97**, 5313-5316.

Titus TA, Larson A (1995) A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae. *Systematic Biology*, **44**, 125-151.

Tortorici L (1981) Analisi delle deformazioni fragili dei sedimenti postorogeni della Calabria settentrionale. *Bollettino della Società Geologica Italiana*, **100**, 291-308.

Uzzell T (1979) Immunological variation in *Rana lessonae* (Amphibia, Salientia). *Mitteilungen aus dem zoologischen Museum in Berlin,* **55**, 29-33.

Uzzell T, Hotz H (1979) Electrophoretic and morphological evidence for two forms of green frogs (*Rana esculenta* complex) in peninsular Italy (Amphibia, Salientia). *Mitteilungen aus dem zoologischen Museum in Berlin,* **55**, 13-27.

Vaccaneo R (1931) Ricerche sui caratteri morfologici dei *Bombinator* italiani. *Boll. Musei Zool.e Anat. comp. R.Univ.Torino*, series III **4**, 1-48.

Van Andel TH, Tzedakis PC (1996) Palaeolithic landscapes of Europe and Environs, 150000 - 25000 years ago. *Quaternary Science Reviews*, **15**, 481-500.

Vanni S, Nistri A (1997) *Salamandrina terdigitata* (Lacepède, 1788). In: *Atlas of amphibians and reptiles in Europe* (eds GASC JP, Cabela A, Crnobrnja-Isailovic J, Dolmen D, Grossenbacher K, Haffner P, Lescure J, Martens H, Martínez Rica JP, Maurin H, Oliveira ME, Sofianidou TS, Veith M, Zuiderwijk A), pp 70-71. Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle, Paris, France.

Veith M (1996) Molecular markers and species delimitation: examples from the European batrachofauna. *Amphibia-Reptilia*, **17**, 303-314.

Veith M, Mayer C, Samraoui B, Donaire Barroso D, Bogaerts S (2004) From Europe to Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed salamanders (genus *Pleurodeles*). *Journal Biogeography*, **31**, 159-171.

Venchi A (2000) *Hyla intermedia.* In: *Anfibi e Rettili del Lazio* (eds Bologna MA, Capula M, Carpaneto GM), pp 38-39. Palombi editore, Italy.

Vila M, Vidal-Romani JR, Bjorklund M, (2005) The importance of time scale and multiple refugia: Incipient speciation and admixture of lineages in the butterfly *Erebia triaria* (Nymphalidae). *Molecular Phylogenetics and Evolution*, **36**, 249-260.

Vitt LJ, Caldwell JP, Wilbur HM, Smith DC (1990) Amphibians as harbingers of decay. *Bioscience*, **40**, 418.

Wallace AR (1869) *The Malay Archipelago* (2 volumes). Macmillan, London.

Webb T, Bartlein PJ (1992) Global changes during the last 3 million years: climatic controls and biotic responses. *Annual Review of Ecology and Systematics*, **23**, 141-173.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population
structure. *Evolution*, **38**, 1358-1370.

Weisrock DW, Macey JR, Ugurtas IH, Larson A, Papenfuss TJ (2001) Molecular phylogenetics and historical biogeography among salamandrids of the 'true' salamander clade: rapid branching of numerous highly divergent lineages in *Mertensiella luschani* associated with the rise of Anatolia. *Molecular Phylogenetics and Evolution*, **18**, 434-448.

Whitlock MC, McCauley DE (1999) Indirect measures of gene flow and migration: F_{ST} not equal $1/(4Nm + 1)$. *Heredity*, **82**, 117-125.

Wright S (1951) The genetical structure of populations. *Annals Eugenics*, **15**, 323-353.

Young AG, Clarke GM, (eds) (2000) *Genetics, Demography and Viability of Fragmented Populations*. Cambridge University Press. Cambridge, UK.

Zhang DX, Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, **12** , 563-584.

Zink RM (2002) Methods in comparative phylogeography, and their application to studying evolution in the North American arid lands. *Integrative and Comparative Biology,* **42**, 953-959.