UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE

Corso di Dottorato di Ricerca in
Ecologia e Gestione delle Risorse Biologiche - XXVI Ciclo

Refuges, divergence and hybridization in naturally fragmented species: the case of the European Plethodontid Salamanders (Genus *Hydromantes*)

(s.s.d. BIO/07)

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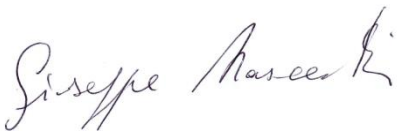

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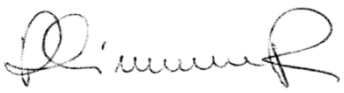

10-06-2014

A Simone, per tutto.

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1. INQUADRAMENTO DELLA RICERCA

Inquadramento della ricerca

Il potenziale evolutivo di una specie è inevitabilmente funzione della sua struttura genetica (Nettel et al., 2008), la quale è il prodotto dell'azione congiunta di fattori di diversa natura (Barton, 2003) come: eterogeneità ambientale, eventi storico-demografici, frammentazione, flusso genico, deriva genetica e ibridazione. Identificare quali tra questi sono maggiormente responsabili della struttura genetica di una data specie è fondamentale per comprendere i processi microevolutivi che hanno determinato la distribuzione geografica delle singole linee intraspecifiche ed il mantenimento dei loro livelli di diversità nel tempo (Knowles and Richard, 2005).

In questo senso, un contributo significativo proviene da casi di studio riguardanti l'analisi filogeografica di specie che presentano dispersione limitata e popolazioni isolate e caratterizzate da una forte frammentazione all'interno dell'areale. Nella letteratura degli ultimi trent'anni è emerso che, in organismi animali così caratterizzati (soprattutto piccoli mammiferi, rettili, anfibi e invertebrati quali artropodi e molluschi), è quasi sempre possibile riscontrare un pattern genetico ricorrente e ben definito. In particolare, tali specie tendono ad avere poche linee evolutive genealogicamente antiche, la cui separazione spesso coincide con eventi paleogeografici e/o paleo-climatici noti. Ciascuna linea evolutiva è rappresentata da popolazioni generalmente differenziatesi in rifugi separati ed è caratterizzata da un set di aplotipi geograficamente ben localizzati (Avise, 2000; Avise, 2009). Il risultato è dunque una forte strutturazione geografica della diversità genetica.

Però, se da un lato le specie caratterizzate da abitudini sedentarie tendono a conservare meglio l'impronta di eventi storico-demografici e biogeografici pregressi, dall'altro, esse hanno spesso popolazioni di piccole dimensioni e risultano quindi vulnerabili agli effetti dei processi casuali di deriva genetica. E' infatti noto che il tasso di deriva aumenta con il diminuire delle dimensioni effettive della popolazione (Crow e Kimura, 1970; Wright, 1931). Ne consegue che, in questi casi, la natura intrinsecamente stocastica di tali processi tende a confondere le tracce di eventi demografici passati, rendendo difficile la ricostruzione della storia evolutiva delle specie in questione, nonché l'interpretazione dei relativi pattern genetici (Koizumi et al., 2012.; Monaghan et al., 2002; Vandergast et al., 2004). Anzi, per alcune delle specie in questione la deriva genetica è stata chiamata in causa come forza predominante nel guidare i processi di diversificazione tra popolazioni e di determinazione della variabilità genetica complessiva (Knowles e Richard 2005; Koizumi et al., 2012). Ciononostante specie poco vagili e con popolazioni piccole e frammentate hanno mostrato, al crescere del numero di casi di studio, di essere in grado di mantenere elevati livelli di variabilità genetica e un chiaro segnale filogeografico, a dispetto dell'azione di forze stocastiche (quali appunto la deriva genetica), che tenderebbero a diluirli.

A tale riguardo, un caso emblematico è quello di *Cambaroides japonicus*, specie dalle abitudini sedentarie, suddivisa in piccole popolazioni isolate all'interno delle sorgenti dei corsi d'acqua in cui vive (Koizumi et al., 2012). I due cladi principali in cui questa specie è suddivisa hanno origine molto antica (Pliocene, 3-5 Ma), ma nonostante ciò la diversità genetica sembra essersi mantenuta nel corso di milioni di anni. Durante le glaciazioni, le popolazioni di questa specie sono sopravvissute all'interno di rifugi centrali dai quali hanno successivamente ricolonizzato le aree circostanti attraverso un processo di tipo stepping-stone. L'attuale assetto genetico della specie è il risultato di un ruolo predominante della deriva che, durante le fasi di ricolonizzazione, agendo sotto forma di effetti del fondatore ripetuti, ha portato alla fissazione di singole mutazioni nei siti colonizzati: l'evidenza principale a supporto di tale scenario è rappresentata dal fatto che le attuali popolazioni sono caratterizzate ciascuna da aplotipi per lo più esclusivi. Tuttavia, nonostante la deriva

genetica in questo caso sia stata chiamata in causa come fattore dominante nel plasmare la struttura genetica di questa specie, essa non è riuscita a cancellare del tutto le tracce antiche del processo graduale che ha portato al differenziamento tra le popolazioni, tanto che la proiezione nello spazio dei rapporti di ancestralità/discendenza tra i vari aplotipi permette di ricostruire chiaramente il processo di colonizzazione stepping-stone. In altri casi invece, la diversità genetica sembra essersi mantenuta attraverso una frammentazione e ripartizione della variabilità ancestrale causata da eventi biogeografici e demografici passati e dalla successiva azione della deriva genetica sulle popolazioni rimaste in condizione di isolamento persistente (Knowles e Richard, 2005). In ogni caso, quello che emerge in questo contesto, è che queste specie costituiscono un vero e proprio paradosso, dal momento che l'elevato grado di frammentazione e la tendenza alla sedentarietà, non hanno impedito il mantenimento di alti o relativamente alti livelli di variabilità genetica nel tempo.

Questi pattern evolutivi unici rendono le specie in questione dei modelli di studio intriganti per osservare gli effetti dell'interazione di fattori di diversa natura sui processi di differenziamento intraspecifici e per meglio comprendere quali dinamiche microevolutive debbano essere mantenute nel tempo, al fine di preservare il potenziale evolutivo di una specie.

Senza dubbio, nell'interpretazione di questi pattern, non si può prescindere dal prendere in considerazione gli effetti degli eventi paleo-climatici che si sono verificati nel tempo. Infatti, come ormai ampiamente dimostrato, l'attuale suddivisione delle linee evolutive intraspecifiche, è in parte il risultato dell'alternanza di fasi glaciali ed interglaciali che si sono succedute nel corso del Quaternario. Benché con conseguenze differenti alle diverse latitudini e sulla base della topografia, queste grandi oscillazioni climatiche hanno avuto effetti diretti sulla maggior parte dei taxa animali e vegetali a livello globale (Jansson and Dyneius, 2002), determinando contrazione ed isolamento delle popolazioni durante i periodi climatici sfavorevoli e successivi eventi di espansione e ricolonizzazione associati a condizioni climatiche più idonee (Hewitt, 1996, 2001, 2004; Schmitt, 2007). Durante gli eventi di espansione, le linee evolutive precedentemente differenziatesi in condizioni di isolamento sono spesso andate incontro a contatti secondari lungo le rotte di dispersione, (Cooper et al., 2011; Devitt et al., 2013 Galbreath et al., 2009; Kropf et al., 2002; Mila et al., 2013; Miraldo et al., 2011; Petit et al., 2003; Shepard e Burbrink, 2009; Zemlak et al., 2008), determinando una riorganizzazione dei genomi e, in alcuni casi, un incremento dei livelli di diversità genetica proprio in corrispondenza delle zone di contatto (Bay et al., 2004; Nettel et al., 2008; Zemlak et al., 2008).

Un gruppo animale che presenta le caratteristiche peculiari appena esposte è rappresentato dai Pletodontidi. Con 27 generi e 440 specie (Amphibia Web, 2014), costituiscono la più ampia famiglia di anfibi urodeli. Nel corso della sua storia evolutiva, questo taxon è andato incontro ad una rapida ed intensa radiazione adattattiva, colonizzando con successo Nord, Centro e Sud America. Da qui, durante periodi di riscaldamento globale, si sarebbe spinto fino in Eurasia (Vieites et al., 2007), dove è attualmente rappresentato solo da due generi, uno asiatico (*Karsenia*) ed uno europeo (*Hydromantes*). Nella regione neartica, dove si concentra quasi l'intera ricchezza di specie della famiglia, questi anfibi presentano spesso concentrazioni sorprendenti di diversificazione a diversi livelli gerarchici e su scale spaziali relativamente ridotte (Garcia-Parìs, 2000). Alle varie latitudini, queste specie si sono adattate ad habitat molto differenti, dalle foreste alle aree sub-desertiche. Nonostante ciò, nel corso del tempo hanno mantenuto una spiccata conservazione di caratteristiche fenotipiche (morfologiche, comportamentali, fisiologiche, dello sviluppo) che si riflette tutt'oggi in una serie di caratteri

comuni all'intero taxon (Wake, 1983). Primi fra tutti, l'elevata filopatria e la bassa vagilità: nel genere *Ensatina*, ad esempio, gli individui si spostano in media non più di 20 m l'anno e nell'arco di diversi anni le distanze massime osservate non superano i 60 m per le femmine e i 120 m per i maschi (Staub et al., 1995); mentre da uno studio condotto su *Plethodon cinereus* è emerso che nell'arco di cinque anni gli individui non si disperdevano oltre i 10 m dal sito natale, con valori medi di 2-4 m per le femmine e 6-8 m per i maschi (Liebgold et al., 2011). Pertanto, gli home range sono piccoli (in genere dell'ordine di pochi mq) e al loro interno i movimenti limitati rendono questi animali per lo più dei "sit-and-wait-foragers" (Cabe et al., 2007; Nishikawa, 1990).

I Pletodontidi sono inoltre caratterizzati dall'assenza di polmoni (caratteristica per altro unica tra i Vertebrati) e, nella maggior parte dei casi, dalla sostituzione dello stadio larvale acquatico con uno sviluppo di tipo diretto. Ciò implica che, sia per la respirazione cutanea sia per la deposizione delle uova, questi organismi necessitano inevitabilmente di un'elevata umidità e di temperature relativamente basse. Tali limiti fisiologici fanno si che il bilanciamento idrico assuma dunque un'importanza cruciale ai fini della sopravvivenza, traducendosi in una stretta associazione a condizioni microclimatiche ristrette e, conseguentemente, in una distribuzione delle popolazioni dettata dall'eterogeneità ambientale. A tale riguardo, un recente studio di Peterman e Semlitsch (2013) sulla salamandra *Plethodon albagula*, ha messo in luce come la distribuzione spaziale dell'abbondanza degli individui e la presenza di stadi diversi del ciclo biologico della specie, fosse tutt'altro che uniforme all'interno dell'areale, bensì plasmata da gradienti ambientali di temperatura e umidità.

Tutte queste caratteristiche appena descritte fanno si che i Pletodontidi abbiano range ristretti con popolazioni frammentate e filogeograficamente ben strutturate, a testimonianza della loro bassa dispersione, ma anche della loro antichità (Garcia-Parìs et al., 2000; Larson et al., 1984; Jockush and Wake, 2002; Pereira e Wake, 2009; Shepard and Burbrink, 2009, Wells, 2007). Ma benché l'antichità delle linee evolutive e la strutturazione genetica dimostrino che questi animali sono sopravvissuti con successo ai grandi eventi climatici del passato (Wake, 2009), rappresentando in taluni casi dei veri e propri relitti pleistocenici (Yanev e Wake, 1981), è ormai ampiamente dimostrato che durante il Quaternario le loro popolazioni sono andate incontro a cambiamenti spaziali e demografici. La bassa dispersione, l'elevata filopatria e l'eterogeneità ambientale hanno fatto si che questi cambiamenti siano avvenuti per lo più attraverso processi di differenziamento a scala locale e frammentazione genetica a scala regionale (Garcia-Paris et al., 2000; Kuchta et al., 2009; Shepard e Burbrink, 2009). Evidenze a riguardo derivano soprattutto da ricerche condotte su Pletodontidi nordamericani del genere *Plethodon*, che attualmente include 55 specie distribuite nelle foreste delle regioni montuose degli Appalachi e delle Interior Highlands. Le specie appartenenti a questo genere sono essenzialmente costituite da popolazioni geograficamente disgiunte su isolati montuosi differenti, separati tra loro da valli a condizioni più xeriche o connessi da stretti passi di alta quota. Sui singoli isolati, specie diverse si sostituiscono l'un l'altra lungo gradienti altitudinali. Sebbene per alcune di queste le oscillazioni climatiche sembrino non aver intaccato la stabilità demografica delle popolazioni (Shepard and Burbrink, 2008), per molte altre specie non è stato così. Queste ultime, seguendo gli spostamenti dei piani vegetazionali, sono andate incontro ad un'alternanza di eventi di contrazione ed espansione soprattutto in senso altitudinale (Shepard and Burbrink, 2009). E' proprio durante le espansioni che si è più volte verificato l'incontro tra unità filogeografiche distinte, a livello sia intraspecifico che inter-specifico (Devitt et at., 2013; Weisrock et al., 2005). I contatti tra linee evolutive diversificatesi in isolamento rappresentano un'occasione per testare se i processi microevolutivi in atto durante le fasi di

divergenza allopatrica abbiano portato o meno al raggiungimento di un isolamento riproduttivo completo. In quest'ottica, i Pletodontidi rappresentano forse uno dei gruppi animali più interessanti da studiare, poiché un aspetto emergente nella letteratura a riguardo è che questi animali hanno avuto e continuano ad avere una storia evolutiva complessa e dinamica, fatta di frequenti interazioni tra popolazioni a diversi stadi di isolamento e speciazione. Uno degli esempi più calzanti a riguardo è quello della specie ad anello *Ensatina eschscholtzii* che in California è presente con un complesso di sette sottospecie distribuite intorno alla zona arida della Central Valley. All'interno di questo range, per lo più continuo, zone di contatto ricorrenti si formano tra linee evolutive a diversi stadi di differenziamento. I processi di diversificazione in questa specie sembrano essere a carico di una stretta interconnessione tra divergenza adattativa, divergenza non adattativa e isolamento storico. In un precedente lavoro (Stebbins, 1949), la suddivisione in sottospecie si è basata sui diversi morfotipi riscontrati, che sembrano essere un adattamento di tipo antipredatorio. Uno studio più recente (Pereira e Wake, 2009), effettuato mediante l'impiego di marcatori nucleari allozimici, ha evidenziato che la transizione da una forma fenotipica all'altra è sempre concordante con le discontinuità genetiche emerse per i dati molecolari. Questo supporta l'ipotesi che ci sia stata senza dubbio una divergenza adattativa e che questa sarebbe stata facilitata da periodi di isolamento, più o meno prolungati, causati sia dai grandi cambiamenti climatici sia dai processi geologici che hanno interessato la regione della Sierra Nevada. In realtà, in questa specie, i periodi di isolamento hanno interessato anche popolazioni morfologicamente simili e geograficamente continue. Ciò ha causato una struttura genetica molto frammentata dove unità evolutive fenotipicamente differenti mostrano livelli di differenziamento pari o inferiori a quelli riscontrati tra unità fenotipicamente criptiche (Kutcha et al., 2009; Wake, 1997). Infatti, attraverso tutto il range della specie, sono presenti zone di ibridazione con alta frequenza di individui ibridi, sia tra popolazioni morfologicamente distinte che tra popolazioni simili. In ogni caso, l'intensità dell'ibridazione è molto più elevata tra popolazioni eco-morfologicamente affini che non tra quelle appartenenti a sottospecie distinte: nelle prime, le zone di contatto arrivano a raggiungere estensioni di quasi 100 km, mentre nelle seconde risultano spazialmente molto più localizzate, estendendosi in media per poco più di 8 km. Quello di *Ensatina* è forse il caso che più esemplifica e sintetizza i meccanismi di azione dei processi di diversificazione tipici dei Pletodontidi, che alternano periodi di isolamento con forte accumulo di divergenza, a episodi di contatto secondario in cui i pool genici diversificatisi in allopatria tornano a fondersi (totalmente o parzialmente, grazie ad una plasticità fenotipica e genetica che permette il flusso genico anche tra taxa mediamente differenziati) generando i livelli particolarmente alti di diversità genetica che caratterizzano queste specie.

Da dati più recenti però inizia ad emergere che anche altri processi contribuiscono agli elevati livelli di diversità riscontrati, primo tra tutti il mantenimento della variabilità genetica primaria delle popolazioni ancestrali. E' questo il caso della specie americana *Batrachoseps major*: in generale, questo pletodontide presenta una forte strutturazione genetica, con livelli complessivamente alti di diversità e aplotipi per lo più esclusivi delle singole località ma, contemporaneamente, le linee evolutive principali continuano a mantenere, nel tempo e nonostante la forte antichità del differenziamento, una serie di polimorfismi ancestrali (Martinez-Solano et al., 2012). Questo tipo di dati dimostra che, anche in specie con popolazioni piccole e frammentate, una parte rilevante della diversità genetica può essere spiegata come diversità primaria, originatasi e mantenutasi in popolazioni isolate, ancorché di piccole dimensioni. Un numero sempre crescente di studi analoghi a quello citato su *B. major* sta rivalutando il ruolo della diversità primaria, dimostrando che anche in specie per le quali il meccanismo del "flush and crash" era ritenuto l'unico vero processo strutturante delle popolazioni (proprio come

nei pletodontidi), l'accumulo di diversità grazie alla persistenza delle popolazioni può essere un meccanismo di rilievo, se non addirittura il principale, nel determinare i livelli di variabilità genetica attuali (Bryson et al., 2014; Byrne, 2008; Byrne e Hopper, 2008; Kuchta et al., 2009; McGiure et al., 2007; Morando et al., 2004; Nevill et al., 2010; Tapper et al., 2014).

I Pletodontidi italiani del genere *Hydromantes*, oggetto del presente studio, sono perfettamente in linea con le caratteristiche generali della famiglia.

Il genere *Hydromantes* è l'unico rappresentante europeo e probabilmente costituisce un relitto di un periodo in cui i Pletodontidi avevano una distribuzione eurasiatica molto più ampia (Wake, 2013: Darlington 1957, p. 163). Particolarità del genere, che ne ha reso in parte difficile la collocazione sistematica, è quella di avere distribuzione disgiunta: tre specie sono endemiche della regione californiana della Sierra Nevada, mentre le altre otto sono esclusive dell'Italia. Di queste ultime, tre hanno una distribuzione peninsulare e le altre cinque sono presenti solamente in Sardegna. L'attuale classificazione divide il genere in tre sottogeneri: *Hydromantes*, *Speleomantes* e *Atylodes* (Wake., 2012). Al sottogenere *Hydromantes* appartengono le tre specie californiane (*H. brunus*, *H. shastae* e *H. platycephalus*); al sottogenere *Speleomantes* appartengono le tre specie italiane continentali (*H. ambrosii*, *H. italicus* e *H. strinatii*) e le quattro specie della Sardegna orientale (*H. flavus*, *H. imperialis*, *H. sarrabusensis* e *H. supramontis*); infine il sottogenere *Atylodes* è monospecifico, poiché include solo la specie *A. genei*, localizzata nella parte sud-occidentale della Sardegna. La separazione dei geotritoni italiani all'interno di due sottogeneri distinti, è stata proposta per dare risalto alle differenze significative tra la specie sud-occidentale sarda e le altre specie insulari e peninsulari. Infatti, non solo i due sottogeneri presentano differenze morfologiche e cariologiche significative, ma sulla base delle ricostruzioni filogenetiche *A. genei* occupa sempre una posizione basale nelle filogenesi, rappresentando quindi il sister taxon delle restanti specie europee di *Hydromantes* (Carranza et al., 2008; van der Mejiden, 2009).

Questi anifibi, comunemente noti come geotritoni, sono animali subtroglofili, caratterizzati da livelli di differenziamento interspecifico variabili. Tra specie insulari e specie continentali i livelli di divergenza sono elevati e anche le distanze genetiche tra coppie di specie sarde sono dell'ordine di valori che nei Pletodontidi risultano con molta probabilità in un isolamento riproduttivo completo (Chiari et al., 2012; van der Meijden et al., 2009). Nel gruppo di specie continentali, invece, i livelli di differenziamento inter- ed intra-specifico sono distribuiti secondo un continuum, piuttosto che in due classi di valori discrete: il grado di divergenza genetica fra cladi della stessa specie e fra specie distinte sono spesso sovrapponibili. Non sorprende, quindi, che casi di ibridazione ed introgressione tra specie distinte siano stati dimostrati in condizioni sia naturali sia artificiali, suggerendo che i contatti secondari tra linee evolutive separate possano aver svolto un ruolo importante nel generare e mantenere la forte variabilità genetica di queste specie (Cimmaruta, 1993; Nascetti et al., 1996).

E' proprio alle tre specie continentali che è stata rivolta l'attenzione in questa ricerca. Di seguito sono riportate le relative distribuzioni geografiche:

Hydromantes strinatii (Aellen, 1958): distribuito nel sud-est della Francia, nella porzione più orientale del "Departement des Alpes-de-Haute-Provence" (Basses-Alpes) e "Departement des Alpes-Maritimes". In Italia è diffuso attraverso tutta la Liguria, arrivando fino alla propaggine nord occidentale dell'Appennino Tosco-Emiliano e toccando le provincie di Cuneo, Alessandria, Massa-Carrara, Pavia, Piacenza e Parma.

Hydromantes ambrosii (Lanza, 1955): occupa un range ristretto, che ricade tra la Liguria e la Toscana, dove è rispettivamente presente con due sottospecie, le cui popolazioni sono geograficamente separate dal fiume Magra. che segna il confine tra la provincia di La Spezia e quella di Massa Carrara. Nello specifico, la sottospecie *H. a. ambrosii* occupa la parte sud-occidentale della provincia di La Spezia, mentre *H. a. bianchii* occupa la parte meridionale della provincia di Massa Carrara.

Hydromantes italicus (Dunn, 1923): presenta un range relativamente ampio con popolazioni distribuite tra l'Appennino Tosco-Emiliano, le Alpi Apuane e l'Apennino centrale (Appennino Umbro-Marchigiano e Appennino Abruzzese).

Nel continuum della distribuzione delle tre specie continentali, *H. ambrosii* occupa una posizione geografica intermedia, poiché essendo distribuito tra la Liguria orientale e la Toscana settentrionale, le sue popolazioni sono parapatriche non solo rispetto a *H. italicus*, ma anche rispetto al geotritone di Strinati. Lavori precedenti (Cimmaruta, 1993; Forti, 1998), hanno evidenziato che in *H. a. ambrosii* sono presenti alleli tipici di *H. strinatii* e *H. italicus*, ma presenti a bassa frequenza in *H. ambrosii*. Sulla base di ciò, era stato ipotizzato che la variabilità del pool genico di questa specie potrebbe essere in parte dovuta all'ibridazione con una delle altre due specie continentali. Per verificare questa ipotesi, e quindi testare il grado di isolamento riproduttivo tra i geotritoni continentali, nel 1983 è stato avviato un esperimento di sintopia artificiale tra *H. a. ambrosii* e *H. italicus* (Forti et al., 2002) dal quale, nell'arco di tredici anni, è emerso che i due taxa presentano un isolamento riproduttivo incompleto, testimoniato dalla formazione di una progenie ibrida con ibridi vitali e fertili. Successivamente all'avvio di questo esperimento, è stata identificata, sulle Alpi Apuane, una zona di ibridazione ed introgressione tra *H. italicus* e la sottospecie *H. a. bianchii* (Cimmaruta et al., 1993; Nascetti et al., 1996; Ruggi et al., 2005). Tra le tre specie, *H. italicus* è quella che mostra la struttura genetica più omogenea, nonché i più alti valori di flusso genico, lasciando ipotizzare che per questa specie l'Appennino non abbia rappresentato una barriera nelle fasi di espansione dell'areale. I livelli di variabilità riscontrati, sulla base di marcatori nucleari allozimici, diventano più alti solo nella parte settentrionale del suo areale, proprio in prossimità della zona di contatto con la sottospecie *H. a. bianchii*. Ciò che si verifica è un'introgressione fortemente asimmetrica, dal momento che mentre gli alleli tipici di *H. italicus* penetrano nel pool genico di *H. ambrosii* per non più di 15 km, l'introgressione di quelli di *H. ambrosii* in *H. italicus* si verifica su un'estensione di circa 90 km (Ruggi et al., 2005). Invece, tra *H. strinatii e H. ambrosii* non è mai stata trovata fino ad oggi nessuna zona di ibridazione, nonostante le due specie vivano in stretta parapatria, con le popolazioni più vicine che distano meno di un km l'una dall'altra (Forti et al., 1997).

In contrasto con gli scenari appena descritti sta il fatto che nessun caso di contatto secondario tra linee evolutive intraspecifiche è mai stato evidenziato all'interno dell'areale di nessuno dei tre taxa in questione.

Rispetto alle altre due specie, nel geotritone di Strinati sono stati riscontrati i livelli di variabilità e frammentazione genetica più elevati (Forti et al., 1997). Poiché questa specie è anche quella che presenta il maggior grado di eterogeneità ambientale all'interno del suo range, sia da un punto di vista orografico che climatico, ci sembra plausibile l'ipotesi che essa possa aver avuto una storia evolutiva particolarmente complessa, durante la quale episodi di isolamento ed espansioni potrebbero essere stati frequenti. Inoltre, parte delle sue popolazioni ricadono nell'area delle Alpi Marittime, al confine tra Francia sud-orientale e Liguria occidentale, nota area di rifugio glaciale (Schönswetter *et al*., 2005), nonché uno dei più importanti hotspot di

biodiversità del Mediterraneo (Médail & Quézel, 1997) sia per gli elevati tassi di ricchezza specifica sia per il numero di endemismi individuati (Casazza et al., 2008). Inoltre il crescente accumulo di studi concernenti specie con popolazioni residenti in questa zona, ha messo in luce come quest'ultima abbia svolto, in alcuni casi, un ruolo di area ancestrale per l'origine di specie e popolazioni (Casazza et al., 2008; Garnier et al., 2004; Grassi et al., 2009; Habel et al., 2005).

Dal momento che le specie in esame si distribuiscono lungo un range geografico praticamente continuo, succedendosi l'un l'altra dalla Francia sud-orientale fino all'Appennino Abruzzese, rappresentano degli ottimi casi di studio, lì dove il fine ultimo è quello di comprendere quali meccanismi concorrono alla generazione e al mantenimento della diversità genetica in presenza di forte frammentazione naturale delle popolazioni.

Il lavoro di tesi si è quindi orientato ad impiegare tecniche molecolari per investigare il ruolo dei diversi pattern e processi di distribuzione della diversità genetica, con particolare riguardo a:

- ruolo dei processi di ibridazione ed introgressione nella generazione della diversità genetica a livello inter- ed intra-specifico, da confrontare con il mantenimento di diversità genetica primaria, in specie caratterizzate da popolazioni piccole ed altamente frammentate;

- influenza degli eventi paleogeografici nel plasmare la struttura genetica in specie legate ad ambienti di rifugio con caratteristiche ambientali costanti (grotte) e caratterizzate da scarsa vagilità.

Gli studi sulla struttura genetica e la filogeografia di *H. strinatii* sono stati condotti con l'obiettivo di ricostruire la storia evolutiva della specie, nonché la distribuzione geografica dei pattern di diversità genetica al suo interno e di verificare se, durante il Pleistocene, le popolazioni sono sopravvissute in condizioni di isolamento in micro-rifugi separati oppure se fluttuazioni cicliche abbiano creato l'opportunità di contatti secondari tra unità evolutive distinte. Nel primo caso ci si aspetta un pattern di forte differenziamento tra genomi tuttora residenti nelle varie aree di rifugio e caratterizzati da una diversità genetica primaria, nel secondo caso dovrebbero essere evidenziabili tracce di contatti secondari o addirittura un rimescolamento ed un'omogeneizzazione del pool genico.

Inoltre, il ruolo dell'ibridazione introgressiva è stato analizzato anche prendendo in esame gli effetti di un esperimento di sintopia artificiale, avviato trent'anni fa, tra *H. a. ambrosii* e *H. italicus* (Forti et al., 2002). L'obiettivo in questo caso è stato quello di valutare il grado di isolamento riproduttivo tra i due taxa e l'eventuale estensione dei meccanismi di ibridazione introgressiva.

I risultati conseguiti sono stati raccolti in due articoli, di cui uno (attualmente in fase di sottomissione) sulla filogeografia di *H. strinatii* dal tilolo "PERSISTENCE, ISOLATION AND DIVERSIFICATION OF A NATURALLY FRAGMENTED SPECIES IN REFUGIAL AREAS" ed un secondo (già pubblicato su *Amphibia-Reptilia*) che riguarda i risultati dell'esperimento di sintopia artificiale dal titolo "THIRTY YEARS OF ARTIFICIAL SYNTOPY BETWEEN *HYDROMANTES ITALICUS* AND *H. AMBROSII AMBROSII* (AMPHIBIA, PLETHODONTIDAE)".

2. PERSISTENCE, ISOLATION AND DIVERSIFICATION OF A NATURALLY FRAGMENTED SPECIES IN REFUGIAL AREAS

Lucente D., Nascetti G. and Cimmaruta R.

Abstract

The study of the phylogeography of the European plethodontid salamander *Hydromantes strinatii* using allozyme and mitochondrial markers allowed recovering a phylogenetic signal despite the stochasticity introduced by the genetic drift due to the small size and the high isolation characterizing the populations of this species. Two highly divergent clades were recovered in the eastern and central-western part of the range, with a further geographic sub-structuring. Nuclear and mt markers recovered substantially the same population groups but were conflicting in reconstructing their relationships. This apparent incongruence highlighted the action of different mechanisms such as incomplete lineage sorting and possible male-mediated gene flow between differentiated lineages. The troglophilic habit of this species provides the opportunity to evidence the importance of caves as microrefugia in maintaining the genetic diversity of this species through persistence of local populations, as highlighted by high nucleotide and haplotype diversity, strong geographic genetic structuring and lack of expansion. This signature was found in the populations from Ligurian and Maritime Alps, in agreement with the complex orography and paleoclomatic history of this Mediterranean hotspot.

Keywords: *Hydromantes strinatii*; Plethodontid salamanders; phylogeography; microrefugia; lineage sorting; secondary contact; Maritime Alps; Ligurian Alps; allozymes; Cytochrome-b cytb; NADH-dehydrogenase subunit 2 ND2

2.1 INTRODUCTION

The role of the Italian peninsula as a refuge during ice ages has been repeatedly evidenced for many different species, with particular regard to the southern area represented by Calabria (Canestrelli et al., 2006, 2008, 2011; Hewitt, 2011). Other northernmost areas such as the Ligurian and French Maritime Alps have been recently pointed out as glacial refugia, at least during the Last Glacial Maximum (LGM) (Casazza et al., 2010; Diadema et al., 2005; Schonswetter et al., 2005). The study effort on this subject is proportional to the relevance of this process with respect to the present patterns of diversity and distribution. Indeed, for many species a significant portion of their current range has been recently recolonized from these refugia, leaving identifiable genetic patterns (i.e. "northern purity-southern richness" and "refugia within refugia"; Gomez and Lunt, 2007; Hewitt, 2004). However, many studies have shown more complex pictures, frequently characterized by discordant mitochondrial (mt) vs. nuclear (nc) patterns, which required complex explanation to be reconciled. These include gene flow between differentiated lineages (either mediated by sex-biased dispersal or not), selective sweeps in mt vs. nc markers, incomplete lineage sorting, long-distance dispersal and/or unusually high migratory rates (Clark et al., 1998; Funk and Homland, 2003; Maddison, 1997; Rheindt and Edwards 2011; Toels and Brelsford, 2012).

More recently, a role for the survival of local populations has been proposed for microrefugia, occurring at a local scale and offering steady and favourable conditions despite the adverse climatic conditions at regional scale (Bryson et al., 2014; Dobrowski, 2011; Mosblech et al., 2011). The idea roots in the study of European forest trees, whose present distribution was hypothesized as due to recolonization from scattered populations based on the finding of higher genetic diversity in northern Alps, i.e. outside the European southern macrorefugia (Birks and Willis, 2008; Petit et al., 2003). The concept was found well fitting to species from topographically complex or fragmented habitats, including Australian granite outcrops and cold-tolerant micromammals (Bhagwat and Willis, 2008; Tapper et al., 2014). Within this context, species linked to subterranean habitats are of particular interest, since caves warrant steady and surface-independent environmental conditions and some data exist proving troglobiont survival in Pleistocene glaciated areas (Allegrucci et al., 2005; Derkarabetian et al., 2010; Hedin, 1997). Even more interesting may be the study of troglophilic species, able to exploit both subterranean and epigean habitats, thus well conforming to the microrefugia model. This postulates that under unfavourable conditions, such as during glacial stages, the species may occupy southern macrorefugia by habitat shift and, at the same time, persist in glaciated areas within hypogean microrefugia. This would allow maintaining unusually high levels of genetic diversity, thanks to the retention of part of the pre-contraction genetic variation at the intrapopulation level and by the maintenance of any new acquired genotype in the microrefugia (Mosblech et al., 2011). Under improved climatic conditions the recolonization process would origin from both micro- and macrorefugia, thus producing a pattern characterized by a particularly high level of diversity and by the presence of highly differentiated populations representing microrefugia (Mosblech et al., 2011). This is for example the case of the North American vaejovid scorpion *Pseudouroctonus reddelli*, showing the expected pattern due to the microrefugia model: high genetic diversity at both nucleotide and haplotype level coupled with high genetic structuring, minimal geographic and population expansion, persistence of local populations even in presence of unfavourable Pleistocenic climatic conditions. The troglophilic habit of the species allowed its survival in caves across its range, where local populations retained genetic diversity instead of losing it through habitat shift/contraction (Bryson et al., 2014).

The importance of caves as microrefugia for troglophilic species deserves deeper analyses, since their role may add to our comprehension of process and mechanism leading to the extant patterns of diversity aside from widely studied macrorefugia. A species of particular interest in this context is the plethodontid salamander *Hydromantes strinatii*, a troghlophilic amphibian inhabiting northwestern Italy and French Maritime Alps (Lanza et al., 2006). *H. strinatii* is lungless, as all *Hydromantes* species, thus relying on cutaneous respiration and so needing steady moist and fresh environments to avoid dehydration and respiratory insufficiency. Moreover these species lack an aquatic larval stage since the metamorphosis is completed within the egg, again requiring a moist, fresh and steady habitats where females can attend to their eggs until hatching occurs (Cimmaruta *et al*., 1999; Lanza *et al*., 1995; Salvidio *et al*., 1994). For these reasons there is a strong association of *H. strinatii* with retreats, as caves and fissure systems, able to warrant the environmental parameters they need during unfavourable climatic periods such as Mediterranean spring and summer. These features make *H. strinatii* a good case study within troglophilic species, since it has some of the traits identifying vertebrate species able to survive in microrefugia such as a small body size (Mosblech et al., 2011). Even more relevant, the strict dependence from underground retreats implies that populations of *H. strinatii* are of small size, naturally fragmented and, due to their low vagility, highly isolated (Lanza et al., 2006). They can be therefore considered "permanently rare species", supposed to have a light genetic load and therefore at lower risk of extinction when constrained in microrefugia (Mosblech et al., 2011). A further reason of interest is linked to the distribution of the species, inhabiting, in the central and western part of its range, the Ligurian and Maritime Alps. This area is characterized by a very complex orography, including karst areas, and by a peculiar paleoclimatic history. The ice sheet which covered the Western Alps during Quaternary glaciations showed a patchy distribution in the coastal zones and many peripheral areas remained free from ice, so constituting refugial micro-areas which resulted in a strikingly high species richness and endemism concentration of plant species (Casazza et al., 2008, 2010; Grassi et al., 2009) as well as a zone of coexistence of *in situ* differentiated lineages in animals (Garnier et al, 2004; Habel et al., 2005; Rousselet et al., 2010).

In this paper we used partial sequences of the mitochondrial genes cytochrome-b (cytb) and NADH-dehydrogenase sub-unit 2 (ND2) and allozyme analysis to study the genetic structure and the phylogeography of *H. strinatii*. This allowed investigating if a phylogeographic signal holds, or is instead overwhelmed by stochastic variation, in a troglophilic species characterized by small population size, low gene flow and pronounced genetic drift. We tested if persistence in microrefugia contributed to present genetic diversity of the species, leaving the expected signature of high haplotypic and nucleotidic diversity associated to strong geographic structuring and to lack of expansion. Also, we tried to assess if the distinctive topography of the Ligurian and Maritime Alps was relevant in determining patterns and processes acting on *H. strinatii* by contrasting the pattern of genetic diversity observed in the populations from this central-western part of the range vs. that observed in the easternmost populations, living in a more homogeneous environment.

2.2 MATERIALS AND METHODS

2.2.1 Sampling and laboratory procedures

An overall number of 385 specimens of *H. strinatii* were sampled from 30 sites distributed throughout the species range (Table 1 and Fig. 1). Standard horizontal starch gel electrophoresis was carried out for all samples using 33 putative allozyme loci, using the laboratory procedures described in detail in Lanza et al. (1995). The following loci were analyzed: 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), Glucose-6-phosphate dehydrogenase (G6pdh; EC 1.1.1.49), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC1.2.1.12,), Superoxide dismutase (Sod-1 and Sod-2; EC 1.15.1.1), Purine nicoside phosphorilase (Np; EC 2.4.2.1), Aspartate transaminase (Aat-1 and Aat-2; EC 2.6.1.1), Creatine kinase (Ck; EC 2.7.3.2), Adenosine kinase (Adk; EC 2.7.1.20), Peptidase C (PepC-2; EC 3.4.11.1), Mannose phosphate isomerase (Mpi; EC 5.3.1.8), Glucose phosphate isomerase (Gpi; EC 5.3.1.9), Glycerol-3-phosphate dehydrogenase (alfa-GPDH; EC 1.1.1.8), 3-hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30), Isocitrate dehydrogenase (Icdh-1 and Icdh-2; EC 1.1.1.42), NADH dehydrogenase (NADH-dh; EC 1.6.99.3), Peptidase-D (Pep-D; EC 3.4.11.1 using Phe-Pro as substrate) Adenosine deaminase (Ada-1 and Ada-2; EC 3.5.4.4), Esterase (Est; 3.1.1.1), Leucine aminopeptidase (Lap; EC 3.4.11.1), Carbonic anhydrase (Ca-2, Ca-3; EC 4.2.1.1), Phosphoglucomutase (Pgm-1 and Pgm-2; EC 5.4.2.2), Fumarase (Fum; EC 4.2.1.2).

In addition, for 194 individuals total genomic DNA was extracted from frozen tissue using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle 1987).

Partial sequences of the mitochondrial genes cytochrome-b (Cytb) and NADH dehydrogenase subunit 2 (ND2) were obtained using specifically designed primers: HST-1f (5'-TTTATTGATTTACCAACCCCATCT-3') and DEB-1r (5'-CAGGGGTGAAGTTTTCTGGAT-3') for Cytb; HND1-f (5'-TCAAGCTTATCCACCGGAAC-3') and MAT2-r (5'-TTGGTGGTAGTCCGCCTAAG-3') for ND2. Amplification was performed by polymerase chain reaction (PCR) in a volume of 25 µl, containing MgCl2 (2.5 mM), the reaction buffer (1X; Promega), the four dNTPs (0.2 mM each), the two primers (0.2 µM each), the enzyme Taq polymerase (2 U; Promega) and 2 µl of DNA template. PCR reactions were carried out in Hain Lifescience Q-Sat 24 thermal cycler using the following program: denaturation at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 s, 53°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 10 min.

Purification and sequencing reactions were outsourced to Macrogen Inc. (www.macrogen.com).

Electropherograms were checked by eye with Chromas v.1.6 (Technelysium Pty Ltd) and sequences were then aligned using the software ClustalX v.1.83 (Thompson et al. 1997).

Two specimens of the closely related species *H. supramontis* and *H. imperialis* were used as outgroups.

2.2.2 Mitochondrial data analysis

Phylogenetic analysis

In order to reconstruct phylogenetic relationships between mitochondrial lineages, we conducted maximum likelihood (ML) and bayesian inference (BI) analyses for the combined dataset of Cytb and ND2 genes, applying in both cases a partition strategy by genes. Sequences of both Cytb and ND2 genes were obtained from Sardinian *Hydromates* species *H. supramontis* and *H. imperialis* and used as outgroups (GenBank accession n. KJ834013-14 and KJ834057-58)

We computed ML analysis in RAxML GUI v.1.3 (Silvestro and Michalak, 2011) using a GTRGAMMA model and setting partitions by codon positions for each gene. The 'ML + thorough bootstrap' option, with one thousand of bootstrap replicates and ten independent searches were implemented and a 50% majority-rule consensus tree was computed. Bayesian inference was carried out using MrBayes v.3.2.2 (Ronquist et al., 2011), specifying different evolutionary models for the distinct codon positions. When the outgroup was excluded, the following optimal models of sequence evolution were selected with jModelTest v.2.1.2 (Darriba et al., 2012) under a Bayesian Information Criterion (BIC) and starting with a ML optimized tree: TPM3uf+I, HKY and TrN respectively for the 1st, 2nd and 3rd codon positions of the Cytb gene; HKY, F81 and TrN+I for the 1st, 2nd and 3rd codon positions of the ND2 gene. We ran three 'heated' and one 'cold' chain for 2 million generations, sampling every 100 generations and discarding 25% of the total sampled trees from the beginning of the chain as burn-in. Standard deviations of split frequencies among chains resulted less than 0.01 and potential scales reduction factors (PSRF) for all parameters were close to one, indicating that convergence was reached. We used Tracer v1.6.0 to check for an acceptable number of independent samples (Rambaut and Drummond, 2007).

We estimated the TMRCA (Time since the Most Recent Common Ancestor) using BEAST 1.8 (Drummond and Rambault, 2007). This analysis was carried out on the topology of the Bayesian tree for the concatenated data set including all the lineages recovered, using the nucleotide substitution model recovered with jModeltest and enforcing a relaxed molecular clock. According to literature, all the Authors agree in dating back the split between eastern Sardinian and mainland lineages to the Messinisn salinity crisis, regardless the markers used in their studies (Carranza et al., 2008; Nascetti et al., 1996). Therefore, the analyses were conducted by constraining the split node between mainland *Hydromantes* species (represented by *H. strinatii*) and Eastern Sardinian species (represented by *H. supramontis* and *H. imperialis*) at 5.33 ma, which is the end of the Messinian salinity crisis (5.96-5.33 ma; Hsu et al., 1973; Krijgsman et al., 1999). Two independent runs were conducted for 20 million generations, sampling every 1000 generations and after discarding the first 20000 steps as burn-in. For each run the achievement of convergence was inspected using the program Tracer 1.6.0 before combining both to estimate the posterior distribution.

Construction of a haplotype network was carried out by median-joining (MJ) algorithm (Bandelt et al., 1999) as implemented in the software Network v.4.6.0 (http://www.fluxus-engineering.com) under Greedy FHP criterion (Foulds et al., 1979) and default parameters values.

Genetic distances between identified groups were computed in MEGA (Tamura et al., 2007) under the Kimura two-parameter (K2P) model (Kimura, 1980).

Population genetic structure and demographic analysis

To investigate population genetic structure we first ran our data in SAMOVA v.1.0 (Spatial Analysis of Molecular Variance; Dupanloup et al., 2002). This method is based on a simulated annealing approach that partitions populations into groups on the basis of geographic proximity and maximization of genetic differentiation. The method needs an a priori range of K values to be set (where K is the number of groups) and then computes, for each K, the amount of genetic variance at the following different hierarchical levels: between groups (F_{CT}), between populations within groups (F_{SC}) and within populations (F_{ST}). The optimum number of groups is inferred looking at the total genetic differentiation between them (F_{CT}), as proposed in Dupanloup et al. (2002). We specifically tested K values ranging from 2 to 25 and performed 100 simulated annealing processes. Additionally, we carried out the identification of groups under a Bayesian clusterization approach, using the software BAPS v.6.0 (Corander et al., 2007, 2008a; Cheng et al., 2013). This method permits to detect optimal clusterization solutions either using a priori geographic information or the only genetic data. In order to observe if there was a convergence of the results, we implemented genetic mixture analyses using the non spatial clustering option. In each case we used the 'Not Fixed K" mode, specifying 20 as the upper limit for the number of clusters and carrying out five replicates for each K.

Once that the best number of groups was selected according to both SAMOVA and BAPS, all the following analyses data were carried out for each of the detected population group.

Haplotype (h) and nucleotide (π) diversity were estimated for each cluster using DNAsp v5.10, as well as the gene flow parameter Nm (Librado and Rozas, 2009).

A hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) was executed in Arlequin v.3.5 (Excoffier et al., 2010) using a pairwise F_{ST} distance matrix and running 1000 permutations.

Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1997) and Ramos-Onsins & Rozas' R2 (Ramos-Onsins and Rozas, 2002) statistical neutrality tests were applied to each group to detect possible historical population growth. Also, we examined distribution of the number of differences between pairs of haplotypes by a mismatch analysis (Slatkin and Hudson, 1991), evaluating both the sum of square deviations (SSD; Harpending et al., 1993) and Harpending's raggedness index (hg; Harpending, 1994) for each group. Tajima's D, Fu's Fs and mismatch distribution were computed in Arlequin v.3.5 (Excoffier et al., 2010), while for R2 computations we used DNAsp v.5 (Librado and Rozas, 2009).

To verify if population genetic structure conformed to an isolation by distance model (IBD), a Mantel test was executed in Arlequin v.3.5 (Excoffier et al., 2010). A genetic F_{ST} distance matrix was performed against a geographic distance matrix (with distances expressed in km), using 10000 permutations. Computations were done for each single group and for the whole populations set.

2.2.3 Allozyme data analysis

Allozyme allele frequencies, conformity to Hardy-Weinberg equilibrium and linkage-disequilibria were calculated using the software Genepop 4.2 (Raymond and Rousset, 1995) testing for departures from expectations using Fisher's method. The parameters of genetic variability per population were estimated using the software Genetix (Belkir et al., 2001): mean number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosisty (H_e), percentage of polymorphic loci under 99% criterion (P_{99}). The software Genodive 2.0 (Meirmans and Van Tienderen, 2004) was used to estimate the effective numbers of alleles (N_e).

The partitioning of genetic diversity was analyzed for variable loci using the Fixation index F_{ST}, as implemented in Genepop 4.2 (Raymond and Rousset, 1995). The significance of pairwaise F_{ST} values was tested using Fisher's method followed by Bonferroni correction (Rice, 1989).

A Principal Component Analysis was carried out using a matrix of covariance of allele frequencies as implemented in Genodive 2.0 (Meirmans and Van Tienderen, 2004) and the significance of the population differentiation represented by the PCA-axes was tested by 100000 permutations.

A Bayesian model-based algorithm was used to assign individuals to genetically homogeneous groups, as implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000). We used an admixture ancestry model assuming correlated allele frequencies and different values of F_{ST} for different subpopulations. Priors were set choosing sampling location information (LOCPRIOR) and using both the mean value of F_{ST} and associated standard deviation obtained by previous calculations. Length of the burn-in period was of 100000 replicates, followed by 100000 Markov chains Monte Carlo (MCMC) replicates and for each K, ranging from 2 to 12, four runs were conducted. The Evanno algorithm (Evanno et al., 2005) was then applied to the results using the online software Structure Harvester v.0.6.93 (Earl and vonHoldt, 2012) to select the optimal K based on both the log probability of the data and the inferred ΔK.

An analysis of molecular variance was carried out assuming a stepwise model as implemented in Genodive 2.0 (Meirmans and Van Tienderen, 2004) partitioning populations according to the K=6 groups recovered by STRUCTURE.

A Mantel test was performed in Arlequin v.3.5 (Excoffier et al., 2010) contrasting a F_{ST} distance matrix vs. a geographic distance matrix (km). The isolation by distance hypothesis was tested for the whole population set, for the two main mitochondrial clades and for the K groups recovered by STRUCTURE.

To identify which loci mostly contribute to the assignment, the program WHICHLOCI was used to rank the scored loci acording to their ability in correctly assign individuals to their population of origin. The programs proceeds through trial assignments determining the assignment efficiency for each single locus (Bank and Olsen, 2003). The analysis was carried out creating 50 synthetic populations through resampling and ranking loci after constraining a minimum percentage of assignment of 95%.

2.3 Results

2.3.1 Mitochondrial data analysis

Sequence variation

The 687-bp fragment of cyt-b sequenced had 59 variable positions (5 singletons) 54 of which were parsimony informative, providing 24 different haplotypes over the 194 specimens scored. The obtained sequences are available in GenBank (Accession n: KJ834015-38). Nucleotide percentages were: T 33.9%, C 22.0%, A 30.4% and G 13.7%. The estimated nucleotide diversity (π) had a mean value of 0.028.

The 702 bp fragment from ND2 gene had 54 variable positions (1 singleton) 53 of which were parsimony informative The 194 specimens scored provided 18 haplotypes, available in GenBank (Accession n: KJ834039-56). Nucleotide percentages were: T 31.2%, C 23.2%, A 35.6% and G 10.0%. The estimated nucleotide diversity (π) had a mean value of 0.025.

The alignment of the concatenated sequences was of 1389 bp, including 113 polimorphic sites, 107 of which were parsimony informative and providing an overall number of thirty haplotypes, as listed in table 1. The estimated values of nucleotide (π) and haplotype (h) diversity were 0.026 and 0.87, respectively. The genetic divergence calculated according to Kimura 2 Parameter (K2P) was 0.028 (±0.003).

Each haplotype was recovered in only one or a few geographically very close localities (Fig. 1).

Phylogenetic analysis

All the phylogenetic reconstructions agreed in showing two well supported reciprocally monophyletic groups, including the haplotypes recovered in the eastern-central and western part of the range, respectively: the eastern clade (Clade A) included the haplotypes recovered from the samples 1 to 21 and the western clade (Clade B) contained those from samples 22 to 30 (Fig. 2). These two clades received a high support for both ML and BI analyses (Fig 2). The Clade A was further subdivided into four geographically distinct sub-clades including the haplotypes from: the easternmost part of the range (sub-clade A1, samples 1-11), the "Rapallo area" (sub-clade A2, samples 12-14), the "Finalese" region (sub-clade A3, samples 15-18), and the "Roburent" region (sub-clade A4, samples 19-21). The branching order within the Clade A was unresolved except for the sub-clade A4, shown as monophyletic and well supported in all the topologies recovered (ML: 85%; BI: 99.6 %). The Clade B was subdivided in three sub-clades each including the haplotypes from a geographic area: "San Bartolomeo Hills" (B1, samples 22-24), "Tenarda" area in the Ligurian Alps (B2, sample 25) and the French Maritime Alps (B3, samples 26-30). The outcome of the median-joining network calculation provided further support to the results of the tree-building methods. We recovered the same two main phylogroups (A and B) separated from a total of 62 steps (Fig 3). Within each phylogroup, the haplotypes were structured in seven geographically distinct haplogroups connected by 7 to 17 mutational steps and completely congruent with the seven sub-clades recovered in the phylogenetic analyses. The sub-clade A1 displayed a starlike pattern, with the haplotype hst1 found at a high frequency in all the samples and representing the 91.5% of its haplotypic diversity. In the sub-clade B3, grouping the haplotypes from the French Maritime Alps, each population was characterized by its own exclusive haplotype(s), with those haplotypes recovered from Tenda (26) showing an intermediate position between the sub-clade B2 (Tenarda, sample 25) and the other haplotypes from French Maritime Alps.

The estimates of K2P distance between the seven lineages gave values comprised between 0.8% and 5.4%. The minimum divergence of 0.8% (p-distance=0.8%) was recovered between the sub-clades A2 and A3, even if they were not the geographically nearest groups. The values of maximum divergence, comprised between 5.0% and 5.4% (p-distance values from 4.7% to 5.2%) were always found in pairwise comparisons between sub-clades A vs. B.

Population genetic structure and demographic history

The SAMOVA analysis showed that the best partitioning of genetic variation was achieved with seven clusters: F_{CT} index grew from 0.79 to 0.95 for K between 2 and 7 and then slightly moved between 0.96 and 0.99 for K between 7 and 25. Accordingly, the algorithm implemented in BAPS recovered 7 as the best group membership solution with a posterior probability of 1.00. The 7 population groups inferred were completely coincident with the seven sub-clades recovered with the phylogenetic analyses, confirming the genetic and geographic pattern of differentiation previously evidenced. The AMOVA analysis was performed on the seven groups recovered by SAMOVA and showed that nearly all the genetic variation was due to the among-group level of variation (95.23%, $P < 0.0001$), while 3.78% was due to the among-population within-group level and 1.00% to the within-population level (Table 2).

The seven clades had heterogeneous levels of both haplotype and nucleotide diversity, with lower values presented by the eastern (A1) and Rapallo (A2) sub-clades (π ranging 0.00004 and 0.00012; h between 0.063 and 0.163) and the highest values recorded for the French clade (B3) with $\pi = 0.00472$ and $h = 0.846$.

Inferred levels of gene flow were very low both between groups (average $Nm = 0.02$) and within groups ($Nm = 0.04$-1.93). Within groups the lowest values of gene flow were estimated within sub-clades A4 and B2.

The Mantel test results showed no correlation between genetic and geographic distances within the seven groups detected. However, a pattern of isolation-by-distance came out when considering the entire species range ($r = 0.52$; $p < 0.0001$). A significant correlation was also observed within the clade A ($r = 0.71$; $p < 0.0001$), conversely clade B exhibited no IBD ($r = 0.71$; $p < 0.0001$).

The demographic test statistics and the analysis of the mismatch distribution computed for each of the seven clades showed only weak signs of past demographic expansion (Table 3). Both Tajima's *D* and Fu's *Fs* gave negative and significant values for the eastern clade A1 and for the S. Bartolomeo clade B1. However, the most sensitive R_2 test did not result in any significant value. On the contrary, the mismatch distribution showed that the frequencies of pair-wise haplotype differences expected under the assumption of a sudden demographic expansion and those observed from the data were similar for all the clades except B3, so that the parametric bootstrap approach did not reject the sudden expansion model for the other 6 clades (A1, A2, A3, A4, B1, B2). However, the curves obtained from observed vs. expected differences were graphically coincident only for clades A1, A2 and B1. Overall, the values obtained testing the demographic history of *H. strinatii* provided weak and sometimes contrasting signals, reasonably supporting a past expansion only for clades A1 and B1.

The chronogram with the obtained confidence intervals (Fig. S1) showed that all the lineages are of Pleistocenic origin, since the split between the two main clades A and B occurred around 1.8 ma (1.82 ± 0.4). Within the clade A the supported note splitting the sub-clade A4 vs. all the others is dated 485000 ya (± 180000) and within the clade b the mains split (B3 vs B1+B2) dated back to 564000ya (± 200000). The differentiation of tip haplotypes occurred between 77000 and 25000 ya.

2.3.2 Allozyme data analysis

Eight loci of the 33 studied resulted monomorphic (*Nadh-dh*, *Np*, *Sod-1*, *Sod-2*, *Adk*, *Lap*, *Fum*, *Mpi*) while the remaining 25 had from two to four alleles per population (Table S1). Twenty private or rare alleles were found, 17 of which were from populations from the Central-Western (CW) part of the range (samples 15-30). The same CW populations showed higher levels of genetic variation than the Eastern (E) samples. The values of the expected heterozigosity H_e ranged from 0.020 to 0.109 in the CW samples and between 0.011 and 0.054 in the E ones; the percentage of polymorphic loci P_{99} was between 6.1 and 39.4 % in CW samples while the E samples provided lower values between 3 and 12.1% (Table 4). Sampled populations were all in Hardy-Weinberg equilibrium except three, showing a heterozygote deficiency at locus *Pep-D*. Linkage disequilibrium was calculated for pairwise loci across all populations and no loci pairs showed significant linkage disequilibrium after Bonferroni correction ($P < 9.5 \times 10^{5}$).

Assignment test using the Bayesian model-based analysis implemented in STRUCTURE subdivided the studied samples in six clusters (K=6) as indicated by both the higher value of ΔK and by the plateau of the estimate Ln probability. The same pattern was recovered carrying out the analysis over the 18 most polymorphic loci alone (data not shown). The six clusters recovered were geographically consistent and largely in agreement with the groups identified by haplotype analyses (Fig. 4). The eastern samples were grouped in two clusters, one comprising samples from 1 to 11 (corresponding to mtA1) and another including samples 12-14 from the "Rapallo area" corresponding to mtA2, with the samples 4, 6 and 11 (SCG, STS, SAL) showing some degree of admixture between these two groups. The samples from the "Finalese" area (15-18) were assigned to a cluster corresponding to mtA3, with nearly no admixture, while those from the "Roburent" area (19-21) formed a fourth cluster corresponding to mtA4. The cluster mtB1 from "San Bartolomeo Hills" was recovered also by STRUCTURE (sample 22 was not scored for allozymes). The sixth cluster was geographically located in the westernmost part of the range, including the samples from Tenarda and the Maritime French Alps (corresponding to clusters mtB2 and mtB3, respectively) and showed various admixture degrees except in populations 28 (SPE) and 30 (SAS). In particular, all the individuals from population 25 (STE) were strongly admixed with clusters A1 and A3. A Principal Component Analysis (PCA) explained 61.0 % of variation with two axes, recovering the larger part of the clusters identified by STRUCTURE (Fig. 5). The only exceptions were the easternmost samples (clusters mtA1 + mtA2), which were lumped together. The samples from the Finalese area (15-18, cluster mtA3), which showed no admixed genotypes, resulted clearly separated while the sample 25 (STE) showed an intermediate position, in accordance with the high level of admixture evidenced by the assignment analysis. The AMOVA carried out taking into consideration the six clusters inferred from STRUCTURE analysis evidenced that the within population and the between clusters levels nearly equally contributed to the total variation (48% and 43%, respectively, $P < 0.0001$ for both), while a small bur significant fraction of total variation was recovered among populations within the groups (9%, $P < 0.0001$).

The software WHICHLOCI was used to identify the loci with the greater assignment power, resulting in a ranking with *α-Gpdh*, *6Pgdh*, *Ada-2*, *Est-4* and *Aat-1* as the top five loci, with scores nearly double than the following three loci (*Mdhp-1*, *Mdhp-2*, *Pgm-1*). The geographic pattern of the allele frequencies at the top five loci evidenced distinct population groups in agreement with the clustering emerging from previous analyses (STRUCTURE, PCA), due to both private alleles and changes in allele frequencies. As an example, the allele frequencies of the locus *α-Gpdh* reported in Fig. 6 showed an eastern group characterized by a widespread

monomorphism (α-*Gpdh*^{85}), while the "Finalese" and "San Bartolomeo Hills" groups were evidenced by nearly exclusive alleles (α-*Gpdh*^{80} and α-*Gpdh*^{75}). The westernmost populations shared the most common allele α-*Gpdh*^{85} but those from "Roburent" area (19-21) were characterized by the presence of the allele α-*Gpdh*^{75}, in common with the nearest group from "Finalese" (15-18), while those from the French Maritime Alps shared the allele α-*Gpdh*^{100} with the far away easternmost group.

The pairwise F_{ST} estimates were always highly significant except when confronting samples from the eastern part of the range (1-11), where only 21 comparisons out of 55 were significant (Table S2). The overall F_{ST} was 0.54 (± 0.066) and the highest values (above 0.70) were recorded when comparing the samples from the "Finalese" (15-18) vs. all the others, with particularly high values vs. the eastern samples (even above 0.80). To investigate the correspondence of population genetic structuring to a model of Isolation By Distance (IBD), F_{ST} and geographic distance values were compared by a mantel test. The results obtained showed a positive correlation across the whole range of the species (r = 0.192, P = 0.0009) as well as within the eastern part of the range (samples 1-14, r = 0.351, P = 0.0080), while IBD does not hold for the western populations.

2.4 DISCUSSION

2.4.1 Population structure of H. strinatii

The results obtained, irrespective of the marker used, evidenced substantial geographical structuring in *H. strinatii*, as expected stated the low vagility and the strong link of this species to the local refuges represented by caves and fissure systems. Large genetic divergence over small geographic scales is quite common in plethodontids, especially if living in geographically old areas (Jockusch and Wake, 2002). However, the geographic pattern of diversity evidenced is not homogeneous across *H. strinatii* range, suggesting different processes leading to the extant eastern (E, 1-14), central (C, 15-21) and western (W, 22-30) population groups. In particular, the E populations are characterized by a low level of genetic diversity, concordantly revealed by both mt and nc markers. A single main haplotype is shared by the larger part of samples within each of the two sub-clades living in this area (A1, A2), together with a few rare singleton differentiated haplotypes. Also, the lowest values for allozyme variability parameters are recorded from these populations, irrespective of the sample size. The pattern of differentiation between populations conforms to IBD model, linking genetic and geographic distances along a NW-SE axis. Since small population size and low gene flow, which are usually invoked to explain low genetic variation (Amos and Harwood, 1998), are typical features of *Hydromantes*, they cannot account for the lower genetic variation of the E vs. CW populations. On the contrary, the signature of an expansion process is present in these populations, having a low genetic variability, a low number of widespread haplotypes and a geographic pattern of genetic diversification following an "expansion route" (Hewitt, 2000, 2004; Ibrhaim et al., 1996). In agreement, the sub-clade A1 (1-11) showed sign of expansion according to neutrality tests and mismatch analysis.

In the C and W parts of the range our data highlighted a higher genetic fragmentation. Nearly each population has its own haplotype and all the F_{ST} values for pairwise comparisons are highly significant. The IBD model does not hold in this area: there is no relationship between genetic and geographic distance, with highly differentiated sample pairs found in the range of a few kilometres. Both nc and mt markers identified 3 groups in the C part of the range, but in the W part the mt data were able to discriminate two distinct sub-clades while allozymes grouped all the W samples in a single cluster. Despite mt and nc data are substantially in agreement in subdividing population groups, the relationships emerging are not concordant. The mt data placed the C samples from the "Finalese" (15-18) in a monophyletic group with the E samples, while the same populations formed the most differentiated group according to allozyme data (Fig. 5). In the W part of the range, the two mt sub-clades were clearly defined while allozymes grouped all W samples in a single cluster characterized by high admixture levels (Fig. 4). In the samples 25, 28 and 29 all the individuals showed a mixed ancestry, with an approximately equal fraction inherited both from the W clade they belong to and from E groups. In sample 25 some degree of admixture with the "Finalese" group was also recorded. This incongruence between mt and nc data can be explained by the "classical" hypotheses of secondary contacts due to sex biased dispersal, which has been frequently used to reconcile contrasting patterns of nc and mt markers in plethodontids. The allopatric divergence is considered as a rule in salamanders since their low vagility promotes genetic fragmentation as soon as a species disperses over a relatively wide area, especially if this is characterized by complex orography and paleogeographic history (Jockusch and Wake, 2002). Higher mutation rate and smaller population size of mt vs. nc genes would account for the deeper differentiation achieved by mt, while the frequent phylopatry of female

salamanders with respect to males (Liebgold et al., 2011) would make nc genes merging faster than mt ones. Thus, in many plethodontids allozymes are supposed to reflect recent and ongoing interactions between taxa and populations (i.e., are able to highlight contact zones and gene flow) while mtDNA bears the signature of ancient, deep events (Jockusch and Wake, 2002).

Here, the clear diversification of mt lineages B2 and B3 (which are paraphyletic with respect to mtB1) is coupled with nuclear admixture in samples 25, 28-30. According to the previously depicted scenario, these mt lineages would reflect a deeper history of differentiation, with more recent secondary contacts involving male biased dispersal, which would be responsible of the admixture observed only at the nuclear markers. This hypothesis however may hold only to explain the admixture of geographically close gene pools. This is the case concerning the presence of the highly divergent "Finalese" genome components (red bars in Fig. 4) into the admixed samples 25 and 19, this latter located less than 10 kilometres far from the nearest sample from "Finalese" group (16) and on the same karstic formation (named "Dolomie di San Pietro ai Monti"). However, the larger part of admixture recorded was due to the presence of common E and W alleles (green bars in Fig. 4) in the gene pool of CW individuals (populations 20-21, 25, 28-30). In this case secondary contacts are unlikely, stated the admixture of geographically distant groups. More relevant, the signature observed in the westernmost part of the range is that typical of the retention of ancestral polymorphism: high genetic variation coupled with the coexistence in the same area of differentiated clades (B2: 28-30; B3: 25; A4: 20-21). Therefore, the sharing of alleles between the W and E populations could be due to incomplete lineage sorting, a result of the coalescent process making alleles present in the common ancestor still retained in descendent lineages. Being it a stochastic process, shared alleles are expected to be randomly distributed in the descendant populations instead of being concentrated along the boundaries between the lineages, as would be the case if secondary contacts would occur (Barbujani et al., 1994; Funk and Omland, 2003).

2.4.2 Evolutionary process and diversification in H. strinatii

The co-occurrence of different evolutionary process in different parts of the range and a peculiar history of diversification, due to the troglophile aptitude of the species, would help explaining the different patterns evidenced by nc and mt markers in *H. strinatii*.

The data presented highlighted high levels of genetic diversity, with most localities having their own haplotypes, and also allozymes were able to evidence highly differentiated populations even over a few kilometres, irrespective to the expected saturation of the signal due to the levels of divergence observed. This was not unexpected, since a strong geographic structure is typical of *Hydromantes* species and related to its ecological and biological features (Carranza et al., 2008; Chiari et al., 2012; Nascetti et al., 1996; Van der Meijden et al., 2009). Both markers evidenced a stronger differentiation in the CW populations with respect to the E sub-clades. The origin of their variation is relatively recent: while the split between the two go back to 1.8 million years ago, diversification within the sub-clades A and B date back to 485 ka and 560 ka years ago, respectively, with the most recent events scattered along a time span covering about 200 ka up to 25-35000 ya. The large HPD intervals prevent the identification of the exact glacial or interglacial events connected to each split, but the overall pattern suggests that transition periods maybe most favourable to these animals than glacial and interglacial stages, being the maximum of both characterized by scarce precipitations (Ivy-Ochs et al., 2006).

This is an unusual pattern, but is in agreement with the features of *Hydromantes*, needing moist and fresh conditions to leave their retreats without suffering a thermal shock (Lanza et al., 2006; Cimmaruta et al., 2005).

Long evolutionary history and large, stable population size are frequently identified as the mechanisms generating and maintaining high genetic diversity, also in widely analysed plethodontid salamanders as for example *Batrachoseps major* (Martínez-Solano et al., 2012). This is not the case of *H. strinatii*, which has very small populations, with a census size in the order of tens of a thousand (Cimmaruta et al., 1999; Salvidio, 2013), and a relatively recent history of diversification when compared to other plethodontids showing similar genetic patterns. However, populations of *Hydromantes* may persist for long time showing a stable demography, despite their small size, thanks to their association with subterranean retreats. The importance of localized refuges in shaping the genetic structure of the species has been recently stressed in many papers regarding a variety of species, from plethodontids (Niemiller et al., 2008) to invertebrates (Allegrucci et al., 2005; Derkarabetian et al., 2010; Snowman et al., 2010) and plants (Tapper et al., 2014). Within this context, the uniqueness of troglophiles species, able to live in both epigean and subterranean habitats, starts emerging, suggesting that these species are able to maintain their small populations stable and safe in the face of paleoclimatic changes (Bryson et al., 2014) and, as a consequence, to retain their own genetic diversity (Mosblech et al., 2011). High haplotype and nucleotide diversity are the signature of historically stable populations and have been recently interpreted as the inheritance of localized refugia, when associated to high geographic structuring and lack of population expansion (Bryson et al., 2014; Mosblech et al., 2011). Our data indicate that the CW clades of *H. strinatii* conform to these expectations having a strong genetic/geographic structuring, high haplotipic and nucleotidic diversity and showing no signs of recent expansion. This witness a prolonged persistence of these populations in their cave retreats, as postulated by the micro-refugia model, but further considerations on the peculiar paleoclimatic history of the area may add to the general picture. Indeed, Ligurian and Maritime Alps are well known glacial refugia, since these areas remained at the borders of the ice sheets during Pleistocene glaciations (Diadema et al., 2005; Minuto *et al*., 2006; Grassi et al., 2009; Zecca et al., 2011). Recent studies have evidenced such kind of glacial refugia both in the Roya Valley (Maritime French Alps) and in the "Finalese", characterized by high levels of both species richness and endemism occurrence and by high genetic variation of local populations, as observed in *H. strinatii* (Grassi et al., 2009; Casazza et al., 2008; Diadema et al., 2005). The bulk of data produced in the last years added a new scenario to the classical one, which interpreted the high genetic diversity of populations from Maritime and Ligurian Alps as due to secondary contacts between Italian peninsular and European lineages (Taberlet, 1998; Hewitt, 2000). According to new data, the Maritime and Ligurian Alps are interpreted as a further glacial refugium and are thus characterized by a high genetic variation of local populations due to the retention of ancestral polyorphism instead of secondary contacts. The data presented on *H. strinatii* support this new scenario.

2.5 CONCLUSIONS

The picture obtained from the study of mt and nc markers in *H. strinatii* provided a complex picture, requiring the action of different evolutionary forces to explain the patterns observed. Despite the low population size typical of this species enhances the effects of the genetic drift, introducing some randomness in the general pattern of variation, and the moderate resolution power typical of allozymes, it has been possible to highlight the complex evolutionary processes in action.

In the CW part of the range the phylogeographic pattern observed are in agreement with a persistence of local populations thanks both to the paleoclimatic history of the Ligurian and Maritime Alps, which remained sheltered from ice cover during the Pleistocene glacial episodes, and to the troglophyle habits of the species, able to use subterranean retreats when climatic conditions become too harsh. In these CW populations therefore the retention of polymorphism through -small- populations stability is the mechanism explaining their high genetic variation, in agreement with the "microrefugia model" (Mosblech et al., 2011). This mechanism can be associated to isolation, therefore producing clearcut differences in allele frequencies, as in the "Finalese". Otherwise signs of nuclear admixture between geographically nearby lineages have been recorded, as in the sub-clade B2 admixed with the neighbouring "Finalese" sub-clade A3, strongly suggesting that rare secondary contacts through male-mediated gene flow may contribute to the maintenance of high genetic variation. Finally, rare long-distance dispersal events took place, leading to the colonization of the eastern part of the range from the CW part. This event is in agreement with the low genetic variability of the E sub-clades (A1, A2) and parsimoniously explains the sharing of alleles, and the consequent admixture detected, with the highly variable W populations.

2.6 ACKNOWLEDGEMENTS

We are indebted to professor B. Lanza, who inspired all our work on *Hydromantes*, besides sampling the French specimens. We are grateful to the many people who helped in collecting the studied specimens and in particular to Dr. G. Forti, Dr. S. Zoia and to the Gruppo Speleologico Imperiese (CAI) for their kind assistance in the field. We thank Dr. D. Canestrelli for his help in the estimation of divergence times and for his useful observations on an early draft of this manuscript.

2.7 TABLES AND FIGURES

Table 1. Sampling localities. Sampling localities with their codes, coordinates, number of individuals analysed using allozymes (N_{nc}) and mtDNA markers (N_{mt}), and the recovered haplotypes.

n°	Locality	Code	Latitude	Longitude	N_{nc}	N_{mt}	mtDNA haplotypes
1	Pietra Vasca Mt. - Genoa, Ligury	SBR	44°15'35.00"	9°30'43.00"	34	5	hSt1 (5)
2	Groppi Mt. - Genoa, Ligury	SBC	44°15'18.00"	9°32'33.00"	8	4	hSt1 (2), hSt3 (2)
3	Environs of Carro - Genoa, Ligury	SSM	44°16'0.00"	9°38'23.00"	10	6	hSt1 (5), hSt4 (1)
4	Pù Mt. - Genoa, Ligury	SCG	44°17'11.00"	9°30'49.00"	8	3	hSt1 (3)
5	Cassagna - Genoa, Ligury	SCA	44°20'16.00"	9°28'1.00"	16	14	hSt1 (12) , hSt2 (1), hSt5 (1)
6	Cave "Tana de Strie" - La Spezia, Ligury	STS	44°19'28"	9°31'9.00"	17	5	hSt1 (5)
7	Cave "Tana da Cruxetta" - La Spezia, Ligury	SCR	44°20'9.00"	9°31'39.00"	8	8	hSt1 (8)
8	Maissana and SW slope of Baralucco Mt. - La Spezia, Ligury	SBN	44°21'9.00"	9°31'47.00"	15	14	hSt1 (14)
9	Codolo - Massa and Carrara, Tuscany	SCO	44°22'05"	9°50'26"	11	-	-
10	Bardi - Parma, Emilia - Romagna	SBD	44°42'28"	09°41'52"	7	-	-
11	Carrega Ligure - Alessandria, Piedmont	SAL	44°36'33.34"	9°10'31.43	10	-	-
12	Rapallo - Genoa, Ligury	SRA	44°20'52.00"	9°12'25.00"	14	1	hSt6 (1)
13	Bargagli - Genoa, Ligury	SBA	44°26'6.00"	9° 3'27.00"	18	15	hSt6 (15)
14	Isoverde - Genoa, Ligury	SIS	44°31'56.00"	8°51'59.00"	24	16	hSt6 (15), hSt7 (1)
15	Millesimo - Savona, Ligury	SOR	44°21'22.00"	8°12'52.00"	5	2	hSt8 (2)
16	Bardineto - Savona, Ligury	SRI	44°11'36.00"	8° 8'52.00"	5	1	hSt8 (1)
17	Finale Ligure - Savona, Ligury	STA	44°12'57.00"	8°21'46.00"	17	11	hSt8 (4), hSt9 (5), hSt10 (1), hSt11 (1)
18	Gavone - Savona, Ligury	SGA	44°10'34"	8°19'35"	5	-	-
19	Toirano - Savona, Ligury	STO	44° 8'16.00"	8°10'09.00"	29	12	hSt14 (12)
20	Rio Roburentello stream - Cuneo, Piedmont	SSL	44°17'41.00"	7°53'25.00"	10	5	hSt13 (5)
21	Roaschia and Rivoera	STR	44°17'23.00"	7°25'49.00"	12	10	hSt12 (5), hSt13 (5)
22	Rezzo - Imperia, Ligury	SRE	44° 1'21.06"	7°52'21.00"	-	3	hSt15 (1), hSt16 (1), hSt18 (1)
23	San Bartolomeo Hill - Imperia, Ligury	SSG	44° 0'4.00"	7°56'13.00"	18	11	hSt15 (11)
24	Cavaronica - Imperia, Ligury	STC	44° 0'12.00"	7°56'46.00"	14	7	hSt15 (6), hSt17 (1)
25	Tenarda - Imperia, Ligury	STE	43°53'30"	7°35'07"	30	24	hSt19 (10), hSt20 (10), hSt21 (1), hSt22 (2), hSt23 (1)
26	Tenda	STN	44° 5'22.79"	7°35'5.57"	10	4	hSt29 (2), hSt30 (2)
27	Luceram	SLU	43°52'31.00"	7°14'50.00"	4	3	hSt26 (3)
28	Peille	SPE	43°48'25.00"	7°15'10.00"	5	2	hSt25 (1), hSt28 (1)
29	Mont Bastide	SMB	43°44'15.36"	7°21'7.47"	8	6	hSt24 (6)
30	Aspremont	SAS	43°46'48.00"	7°14'31.00"	13	2	hSt27 (2)

Tab.2. Analysis of molecular variance (AMOVA) for mitochondrial data. Hierarchical subdivision of genetic variance among the seven mitochondrial groups identified by phylogenetic analyses.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among the 6 groups	6	3560.285	21.96918	95.23
Among populations within groups	19	103.478	0.87103	3.78
Within populations	168	38.65	0.23006	1.00
Total	193	3702.413	23.07027	

Fixation Indices

F_{SC} : 0.79106 P-value =0.0000

F_{ST} : 0.99003 P-value =0.0000

F_{CT} : 0.95227 P-value =0.0000

Table 3. Results of genetic diversity estimates, neutrality tests and mismatch distribution for mitochondrial data. For each of the seven mitochondrial groups identified with phylogenetic analyses are reported: number of individuals analysed, haplotype diversity (Hd), nucleotide diversity (π); values of Tajima's D, Fu's and R2 tests; sum of squared deviations (SSD) and raggedness index (r). *P*-values associated to neutrality tests and mismatch distribution statistics are also reported.

							Demographic expansion		**Spatial expansion**	
	N	**Hd**	**π**	**Tajima's D test**	**Fu's FS test**	**R2**	**SSD**	**r**	**SSD**	**r**
Orientali	59	0.163	0.00012	-1.762	-5.228	0.0555	0.00068	0.48452	0.00068	0.48452
P				*0.005*	*0.001*	*0.1577*	*0.34*	*0.7*	*0.68*	*0.4*
Isoverde	32	0.063	0.00004	-1.14244	-1.264	0.16132	0.00001	0.76953	0.00001	0.76953
P				*0.134*	*0.003*	*0.677*	*0.21*	*0.83*	*0.18*	*0.85*
Tascea	14	0.659	0.00057	0.7	-0.929	0.18	0.02892	0.20879	0.02892	0.20879
P				*0.802*	*0.135*	*0.63*	*0.11*	*0.11*	*0.06*	*0.1*
Tenarda	24	0.67	0.00128	0.944	0.636	0.1739	0.07349	0.21333	0.05312	0.21333
P				*0.86*	*0.676*	*0.8517*	*0.2*	*0.18*	*0.18*	*0.47*
Toirano	27	0.655	0.00304	2.566	7.779	0.2343	0.17749	0.45163	0.10843	0.45163
P				*0.996*	*0.995*	*1*	*0.07*	*0*	*0.1*	*0.35*
Sgarbu	21	0.271	0.00021	-1.726	-2.819	0.1166	0.00493	0.28143	0.0011	0.28143
P				*0.019*	*0*	*0.17045*	*0.42*	*0.58*	*0.48*	*0.57*
Francesi	17	0.846	0.00472	0.43	2.057	0.1615	0.05163	0.12084	0.03707	0.12084
P				*0.723*	*0.839*	*0.744*	*0.01*	*0.01*	*0.33*	*0.56*

Table 4. Genetic variability parameters calculated over the 33 allozyme loci scored in *H. strinatii*. Per each sample are reported: the mean (N_a) and effective (N_e) number of alleles; the observed (H_o) and expected heterozygosity (H_e); the percentage of polymorphic loci with the 99% criterion (P_{99}).

Population	Code	N_a	N_e	H_o	H_e	P_{99}
1	**SBR**	**1.182**	**1.02**	**0.013**	**0.017**	**0.152**
				0.038	*0.05*	
2	**SBC**	**1.121**	**1.087**	**0.068**	**0.054**	**0.121**
				0.214	*0.155*	
3	**SSM**	**1.061**	**1.032**	**0.027**	**0.022**	**0.061**
				0.11	*0.091*	
4	**SCG**	**1.121**	**1.044**	**0.022**	**0.03**	**0.091**
				0.074	*0.113*	
5	**SCA**	**1.091**	**1.013**	**0.012**	**0.011**	**0.091**
				0.049	*0.044*	
6	**STS**	**1.091**	**1.036**	**0.007**	**0.022**	**0.091**
				0.032	*0.092*	
7	**SCR**	**1.061**	**1.033**	**0.018**	**0.022**	**0.061**
				0.071	*0.092*	
8	**SBN**	**1.121**	**1.037**	**0.032**	**0.026**	**0.121**
				0.111	*0.085*	
9	**SCO**	**1.091**	**1.036**	**0.015**	**0.024**	**0.091**
				0.066	*0.09*	
10	**SBD**	**1.03**	**1.015**	**0.013**	**0.011**	**0.03**
				0.075	*0.063*	
11	**SAL**	**1.061**	**1.034**	**0.022**	**0.024**	**0.061**
				0.089	*0.085*	
12	**SRA**	**1.121**	**1.07**	**0.024**	**0.038**	**0.091**
				0.088	*0.133*	
13	**SBA**	**1.061**	**1.033**	**0.025**	**0.02**	**0.061**
				0.113	*0.089*	
14	**SIS**	**1.091**	**1.044**	**0.017**	**0.028**	**0.091**
				0.081	*0.101*	
15	**SOR**	**1.152**	**1.071**	**0.053**	**0.049**	**0.151**
				0.131	*0.142*	
16	**SRI**	**1.25**	**1.133**	**0.086**	**0.081**	**0.219**
				0.178	*0.176*	
17	**STA**	**1.182**	**1.079**	**0.049**	**0.051**	**0.151**
				0.13	*0.148*	
18	**SGA**	**1.182**	**1.097**	**0.059**	**0.055**	**0.151**
				0.157	*0.144*	
19	**STO**	**1.455**	**1.156**	**0.093**	**0.083**	**0.394**
				0.169	*0.157*	
20	**SSL**	**1.156**	**1.075**	**0.048**	**0.045**	**0.156**
				0.132	*0.126*	
21	**STR**	**1.062**	**1.031**	**0.021**	**0.02**	**0.061**
				0.083	*0.083*	
23	**SSG**	**1.364**	**1.194**	**0.112**	**0.079**	**0.333**
				0.191	*0.135*	
24	**STC**	**1.333**	**1.18**	**0.105**	**0.104**	**0.272**
				0.191	*0.193*	
25	**STE**	**1.394**	**1.162**	**0.092**	**0.089**	**0.242**
				0.181	*0.176*	
26	**STN**	**1.242**	**1.125**	**0.083**	**0.094**	**0.242**
				0.168	*0.19*	
27	**SLU**	**1.188**	**1.126**	**0.079**	**0.083**	**0.182**
				0.181	*0.194*	
28	**SPE**	**1.125**	**1.09**	**0.083**	**0.109**	**0.151**
				0.225	*0.288*	
29	**SMB**	**1.125**	**1.074**	**0.077**	**0.075**	**0.151**
				0.212	*0.217*	
30	**SAS**	**1.273**	**1.157**	**0.093**	**0.07**	**0.212**
				0.19	*0.156*	

Figure 1. Distribution map of the 30 sampling sites of *Hydromantes strinatii*. Further information about sampled localities are given in Table 1. Colours denote the seven sub-clades recovered by phylogenetic analyses, with the exception of black populations that were included only in allozyme analysis.

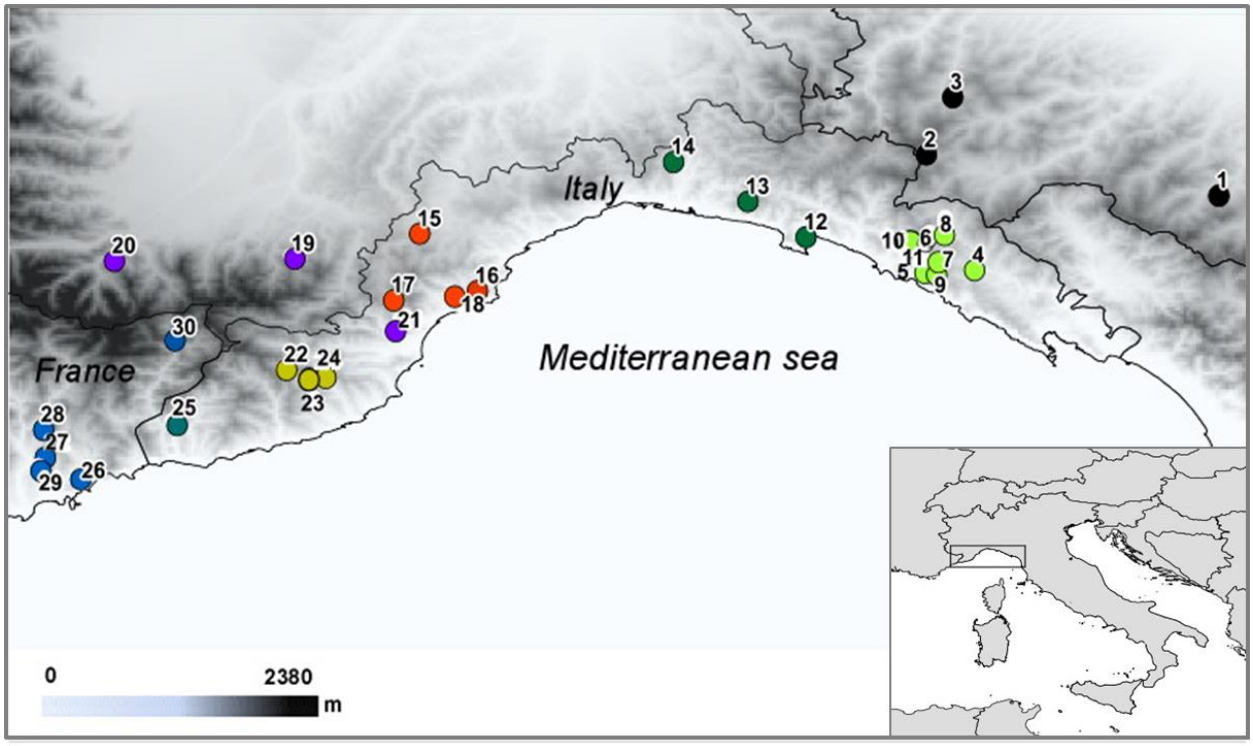


Figure 2. Phylogenetic relationships between mtDNA haplotypes. The tree shows the results for Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. Posterior probability and bootstrap values are reported on each tree node. Both phylogenetic reconstructions showed the occurrence of two main clades A and B, subdivided respectively into four (A1, A2, A3, A4) and three (B1, B2, B3) sub-clades.

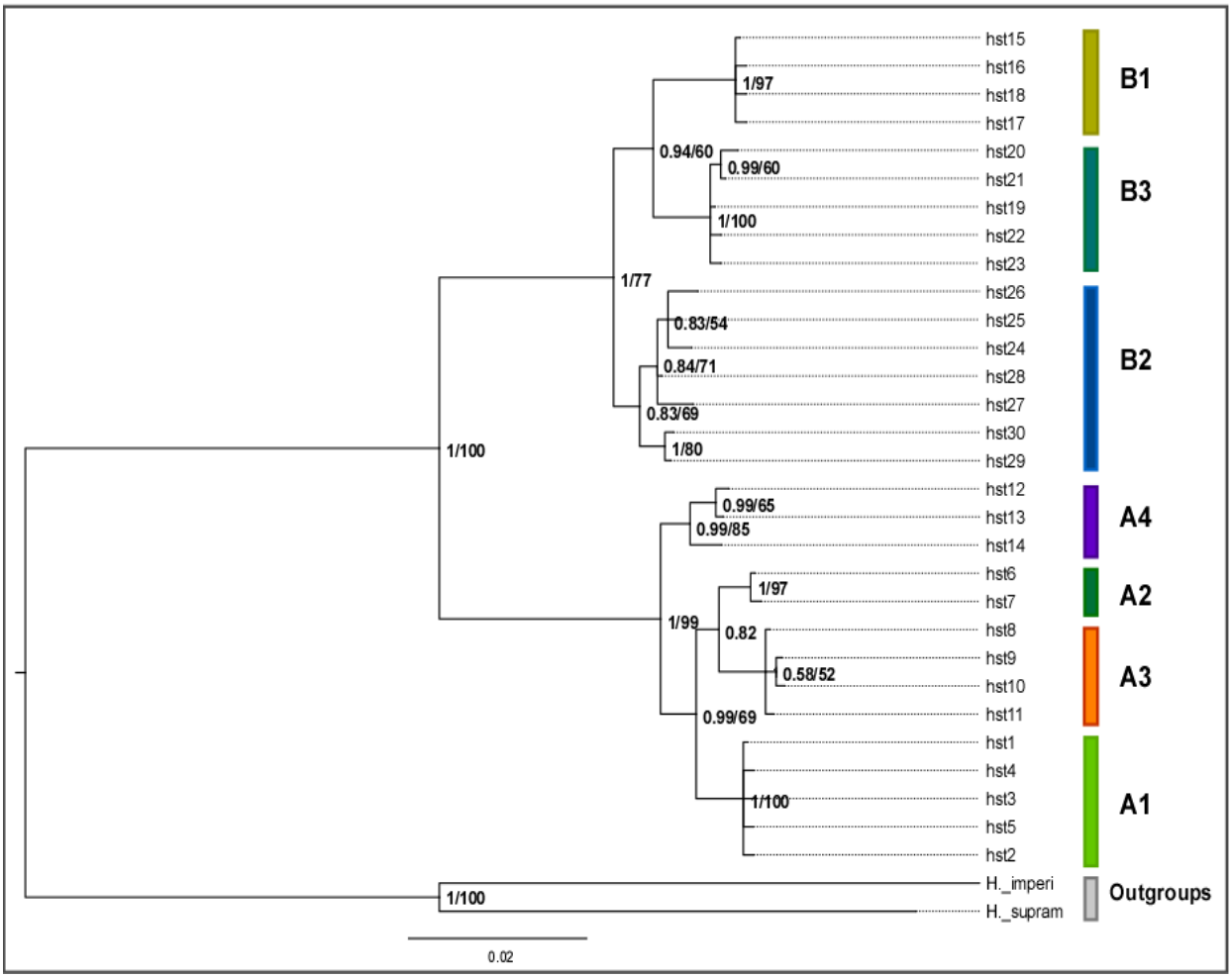


Figure 3. Median joining haplotype network based on the 194 mitochondrial concatenated sequences of Cytb and ND2 genes. The size of the circles is proportional to the frequency of a haplotype across the sampled individuals. The haplotypes are connected by distances proportional to mutational steps, with the small grey circles representing missing haplotypes. The seven haplogroups are congruent to the seven sub-clades recovered by phylogenetic analysis; the two main clades A and B are separated by 62 mutational steps.

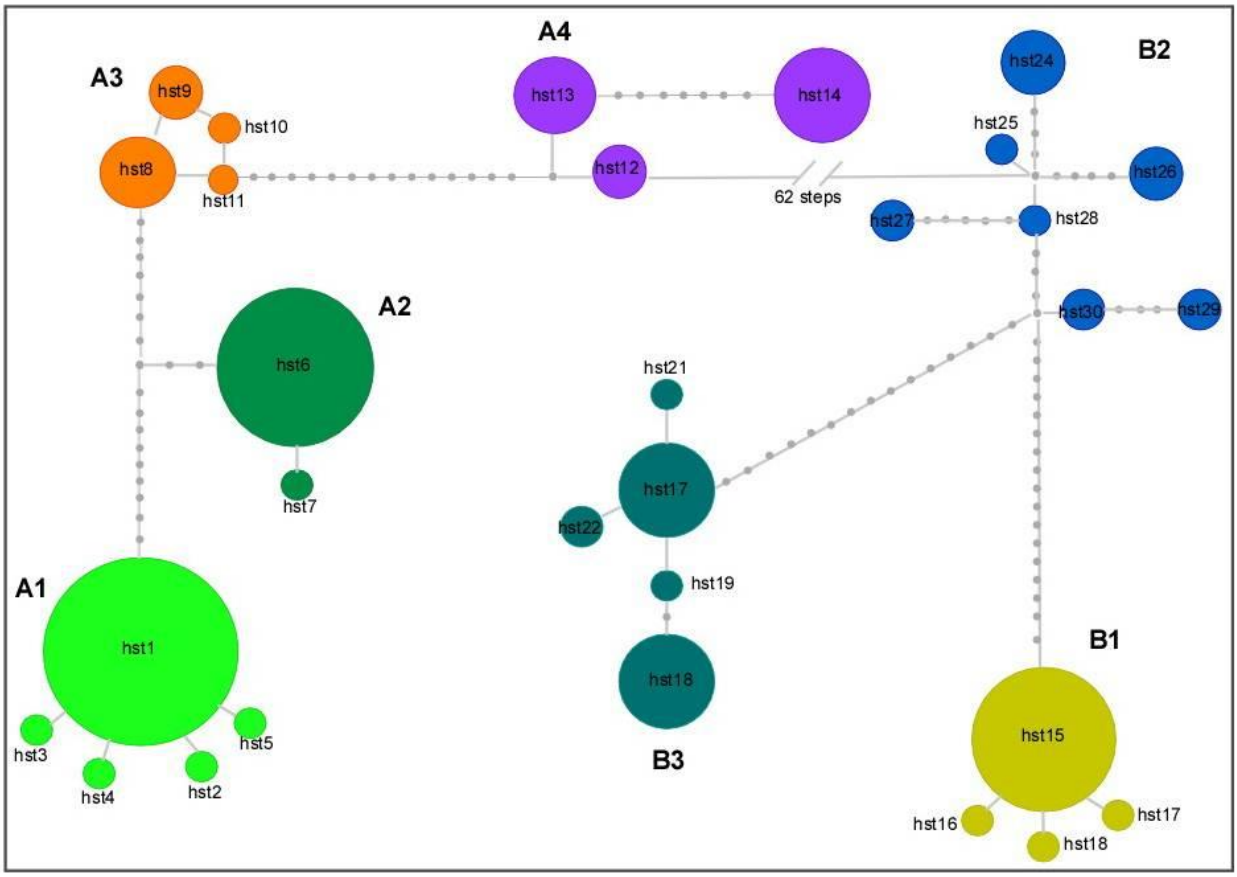


Figure 4. Assignment of *H. strinatii* individuals based on allozyme data. Assigment test of specimens (one per column) to the K=6 clusters identified by the software STRUCTURE based on the 33 scored allozyme loci. Specimens having admixed genomes are identified by columns showing different colours, proportional to the percentage of the genome assigned to each cluster.

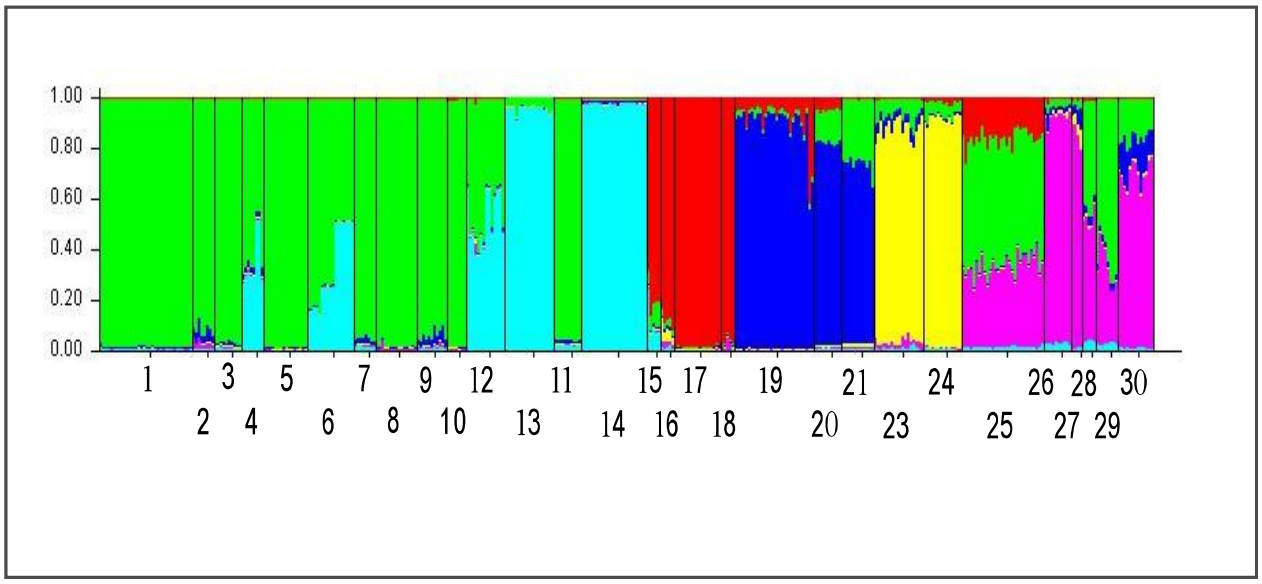


Figure 5. Principal Component Analysis of allozyme allele frequencies in *H. strinatii*. Population groups are identified using the same colors used in Fig. 1, except for sample 25 (STE) which has been leaved unmarked.

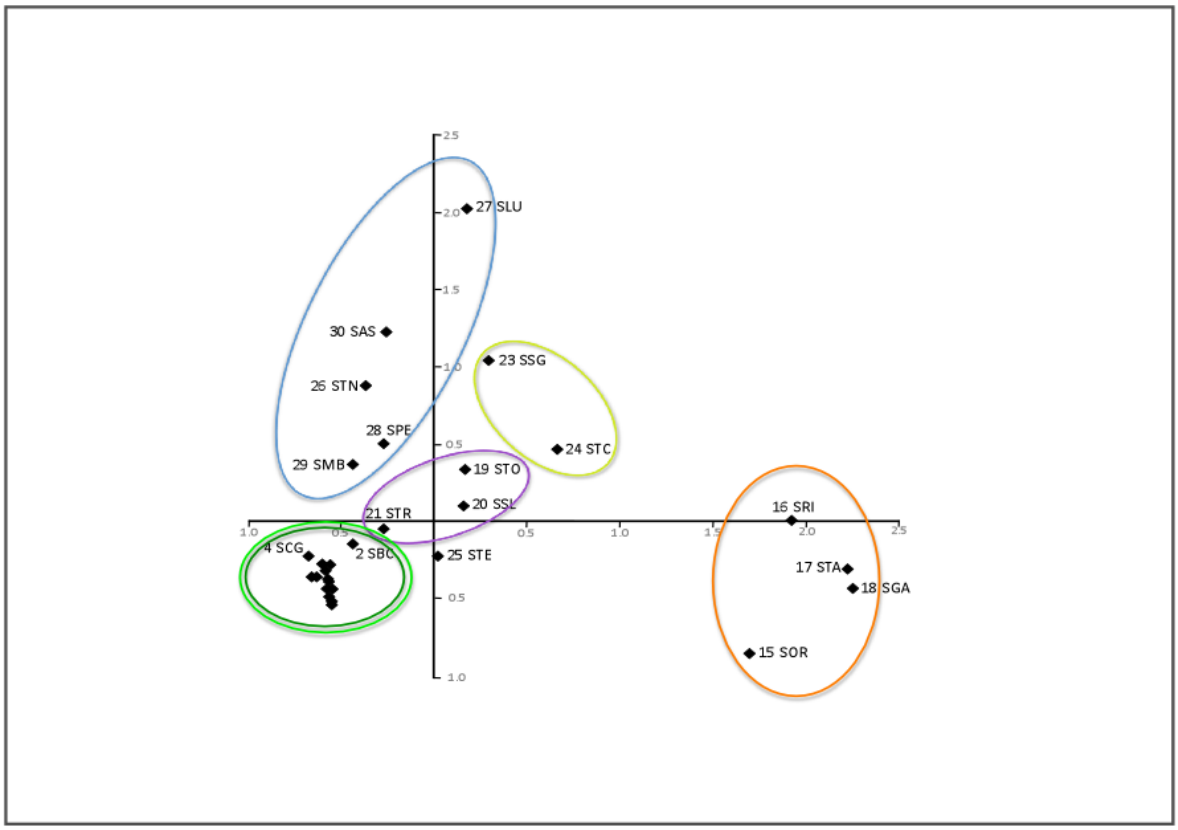


Figure 6. Geographic pattern of the allele frequencies at the allozyme locus *a-Gpdh*.

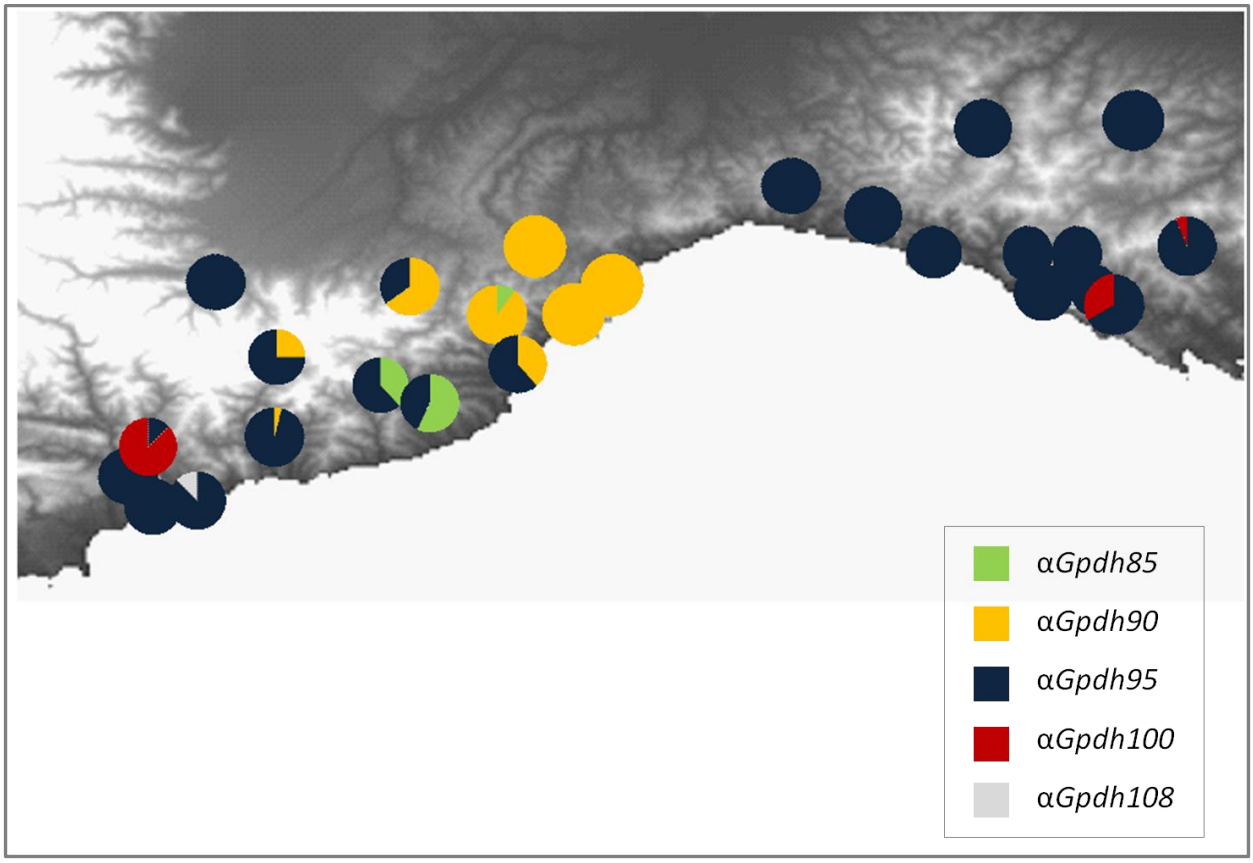


Table S2. Pairwise fixation index (F_{ST}) values based on allozyme data. * = P<0.05; **P<0.01; ***P<0.001; ---not significant

Pop	1-SBR	2-SBC	3-SSM	4-SCG	5-SCA	6-STS	7-SCR	8-SBN	9-SCO	10-SBD	11-SAL	12-SRA	14-SBA	13-SIS	15-SOR	16-SRI	17-STA	18-SGA	19-STO	20-SSL	21-STR	23-SSG	24-STC	25-STE	26-STN	27-SLU	28-SPE	29-SMB
2	0.2793**																											
3	0.0536–	0.0751–																										
4	0.3447*	0.2704*	0.2425*																									
5	0.0074–	0.2835**	0.0507–	0.4417***																								
6	0.2586***	0.3164***	0.1905**	-0.0733–	0.3330***																							
7	0.0342–	0.1177–	-0.0278–	0.1979–	0.1299–	0.1842***																						
8	0.1027–	0.1313–	0.0030–	0.3184**	0.0377–	0.2894***	0.0862–																					
9	0.0639–	0.1388–	-0.0012–	0.2032*	0.1626–	0.2137***	-0.0853–	0.1283*																				
10	0.0229–	0.2054*	0.0109–	0.4402**	-0.0281–	0.3252**	0.1212–	-0.0051–	0.1459–																			
11	0.0957–	0.0643–	-0.0619–	0.2014–	0.0510–	0.1657*	-0.0179–	-0.0160–	0.0223–	-0.0185–																		
12	0.2017***	0.2920***	0.1950**	0.1333–	0.2788***	0.1518**	0.1450**	0.2507***	0.1776***	0.2339**	0.2343***																	
13	0.6071***	0.5692***	0.5654***	0.3764**	0.6729***	0.4336***	0.5743***	0.5964***	0.5738***	0.6983***	0.2508***	0.5475***																
14	0.5803***	0.5301***	0.5383***	0.4206***	0.6446***	0.4969***	0.4987***	0.5729***	0.5193***	0.6136***	0.2417***	0.0836***	0.5679***															
15	0.7619***	0.6256***	0.6957***	0.7157***	0.7643***	0.7227***	0.6831***	0.6989***	0.6962***	0.7266***	0.6537***	0.7685***	0.7188***	0.744***1														
16	0.8206***	0.6332***	0.7100***	0.7178***	0.7745***	0.8063***	0.7064***	0.7360***	0.7583***	0.7062***	0.7332***	0.7638***	0.7358***	0.8152***	0.2698–													
17	0.8120***	0.7171***	0.7558***	0.7874***	0.7954***	0.7966***	0.7320***	0.7756***	0.7624***	0.7827***	0.7688***	0.8098***	0.7661***	0.8099***	0.2485**	0.1735**												
18	0.8256***	0.6805***	0.7678***	0.7720***	0.8094***	0.8010***	0.7345***	0.7489***	0.7648***	0.7609***	0.7399***	0.8250***	0.7803***	0.8067***	0.2600–	0.1446–	0.0034–											
19	0.4289***	0.2707***	0.2870***	0.2526***	0.4340***	0.3812***	0.2511***	0.4141***	0.2900***	0.3867***	0.3606***	0.4701***	0.2644***	0.4648***	0.5047***	0.4236***	0.5490***	0.5431***										
20	0.5137***	0.3037***	0.3687***	0.3985***	0.4847***	0.5440***	0.3934***	0.4339***	0.4517***	0.4165***	0.5128***	0.5457***	0.3968***	0.6477***	0.5883***	0.5328***	0.6267***	0.6167***	0.1757***									
21	0.5963***	0.3892***	0.4553***	0.4513***	0.5264***	0.5536***	0.4783***	0.4209***	0.5060***	0.4744***	0.5255***	0.6204***	0.4385***	0.6780***	0.7319***	0.7210***	0.7624***	0.7534***	0.2943***	0.2744***								
23	0.5211***	0.2946***	0.3606***	0.3809***	0.4738***	0.4876***	0.3848***	0.4517***	0.4165***	0.3699***	0.4590***	0.5243***	0.3613***	0.5577***	0.4928***	0.4523***	0.5566***	0.4971***	0.3065***	0.2952***	0.2991***							
24	0.5516***	0.3312***	0.3788***	0.4738***	0.5259***	0.5397***	0.4396***	0.5058***	0.4618***	0.4097***	0.5027***	0.5862***	0.4072***	0.6091***	0.3811***	0.4426***	0.5064***	0.4448***	0.3900***	0.4057***	0.4650***	0.1105***						
25	0.2643***	0.2052***	0.1821***	0.2160***	0.1868***	0.2292***	0.1607***	0.1751***	0.1934***	0.1548***	0.2327***	0.3783***	0.1867***	0.4200***	0.4002***	0.4377***	0.5364***	0.4451***	0.2484***	0.2791***	0.2605***	0.3261***	0.3779***					
26	0.7356***	0.5517***	0.6149***	0.5679***	0.7027***	0.6381***	0.6054***	0.6375***	0.6427***	0.6453***	0.6210***	0.6560***	0.6060***	0.6890***	0.6835***	0.6396***	0.7352***	0.7006***	0.5477***	0.4594***	0.5406***	0.3866***	0.4692***	0.4209***				
27	0.7956***	0.5140***	0.6602***	0.6776***	0.7773***	0.7722***	0.6786***	0.7244***	0.7221***	0.7032***	0.7048***	0.7746***	0.6925***	0.7973***	0.6915***	0.5616***	0.7256***	0.6646***	0.4345***	0.5200***	0.6744***	0.2359***	0.4108***	0.4527***	0.4890***			
28	0.6548***	0.4425***	0.5058***	0.5100***	0.5961***	0.5633***	0.5038***	0.5315***	0.5336***	0.5448***	0.4667***	0.6546***	0.5463***	0.6863***	0.6663***	0.5528***	0.7187***	0.6768***	0.3011***	0.5182***	0.6146***	0.3639***	0.4739***	0.2275***	0.5915***	0.4494***		
29	0.4572***	0.2619***	0.2777***	0.3268***	0.4165***	0.3547**	0.3228***	0.3376***	0.3196***	0.3662***	0.2997***	0.5306***	0.3439***	0.5896***	0.6580***	0.5949***	0.7426***	0.7095***	0.2658***	0.3729***	0.4125***	0.3370***	0.4543***	0.1853***	0.5429***	0.4966***	0.1694–	
30	0.5781***	0.3939***	0.4414***	0.3985***	0.5031***	0.4934***	0.4123***	0.4725***	0.4470***	0.4545***	0.4752***	0.5627***	0.4324***	0.5961***	0.6058***	0.5664***	0.6755***	0.6046***	0.3100***	0.3787***	0.3630***	0.2335***	0.4110***	0.2743***	0.4330***	0.2141***	0.1498**	0.2450***

Table S1. Allozyme allele frequencies. Allozyme allele frequencies per population at the 25 polymorphic loci out of the 33 scored.

	Sample														
Locus	SBR	SBC	SSM	SCG	SCA	STS	SCR	SMV	SCO	SBD	SAL	SRA	SBA	SIS	SOR
Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
a-Gpdh															
85	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
90	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000
95	1.000	0.667	1.000	1.000	1.000	1.000	1.000	1.000	0.938	1.000	1.000	1.000	1.000	1.000	---
100	---	0.333	---	---	---	---	---	---	0.062	---	---	---	---	---	---
108	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ldh-1															
97	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.938	1.000	1.000	1.000	1.000	1.000	1.000	1.000
107	---	---	---	---	---	---	---	0.062	---	---	---	---	---	---	---
Ldh-2															
108	1.000	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
112	---	0.125	---	---	---	---	---	---	---	---	---	---	---	---	---
Hbdh															
105	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
120	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Mdh-1															
90	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-2															
107	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
115	1.000	1.000	1.000	1.000	0.969	0.971	1.000	0.900	1.000	1.000	1.000	1.000	1.000	1.000	1.000
125	---	---	---	---	0.031	0.029	---	0.100	---	---	---	---	---	---	---
Mdhp-1															
110	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
118	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
126	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Mdhp-2															
94	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900	1.000	1.000	1.000
103	---	---	---	---	---	---	---	---	---	---	---	0.100	---	---	---
Idh-1															
90	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
94	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
103	0.017	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Idh-2															
97	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
110	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
6Pgdh															
87	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
95	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
97	0.939	0.500	0.750	0.900	0.867	0.912	0.875	0.700	0.909	0.786	1.000	0.900	0.700	0.947	1.000
103	0.061	0.500	0.250	0.100	0.133	0.088	0.125	0.300	0.091	0.214	---	0.100	0.300	0.053	---
Gapdh															
93	0.981	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
96	0.019	0.825	---	---	---	---	---	---	---	---	---	---	---	---	---
105	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Aat-1															
90	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
103	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000
110	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	---
120	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Aat-2															
88	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
105	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ck															
90	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Follows

Following

Table S1. Allozyme allele frequencies

	Sample														
Locus	SBR	SBC	SSM	SCG	SCA	STS	SCR	SMV	SCO	SBD	SAL	SRA	SBA	SIS	SOR
Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pgm-1															
92	---	---	---	---	---	---	---	---	---	---	0.385	0.625	---	0.771	0.300
102	0.912	1.000	1.000	0.962	1.000	1.000	1.000	1.000	1.000	1.000	0.615	0.375	1.000	0.229	0.700
110	0.088	---	---	0.038	---	---	---	---	---	---	---	---	---	---	---
Pgm-2															
86	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
92	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Est-4															
95	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
105	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.200
110	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800
Ap															
95	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.200
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800
Pep-D															
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
110	0.958	0.800	0.800	0.833	1.000	1.000	0.667	0.933	0.615	1.000	0.875	0.833	0.833	0.833	1.000
115	0.042	0.200	0.200	0.167	---	---	0.333	0.067	0.385	---	0.125	0.167	0.167	0.167	---
125ß	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ada-1															
85	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
98	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ada-2															
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
103	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.900
106	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
110	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.100
Ca-2															
90	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ca-3															
82	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
90	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Gpi															
93	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
95	---	---	---	0.100	---	---	---	---	---	---	---	---	---	---	---
100	1.000	1.000	1.000	0.900	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Follows

Following

Table S1: Allozyme allele frequencies

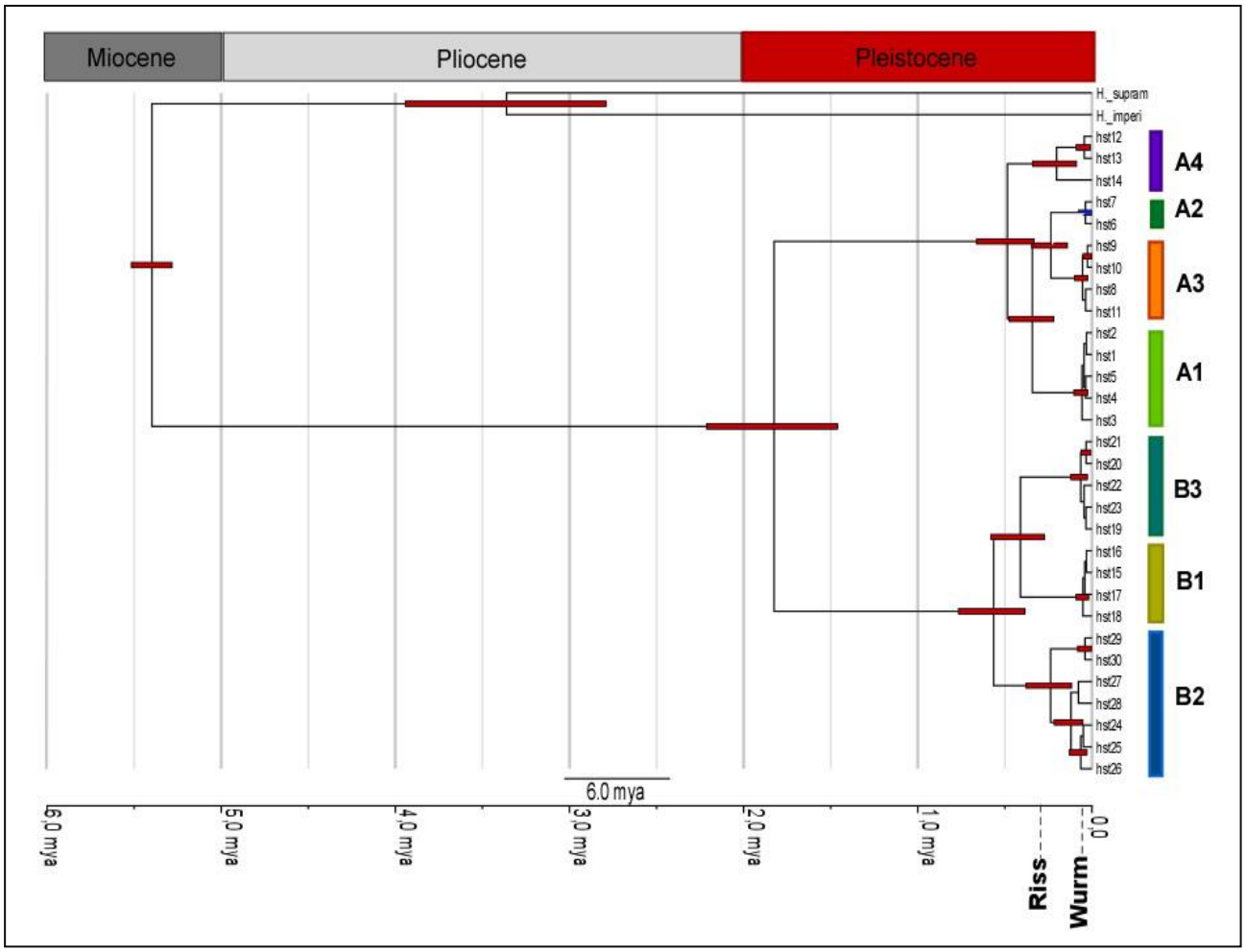
	Sample													
Locus	SRI	STA	SGA	STO	SSL	STR	SSG	STC	STE	STN	SLU	SPE	SMB	SAS
Allele	16	17	18	19	20	21	23	24	25	26	27	28	29	30
a-Gpdh														
75	0.100	---	---	---	---	---	0.382	0.571	---	---	---	---	---	---
80	0.900	1.000	1.000	0.385	0.650	---	---	---	0.038	0.250	---	---	---	---
85	---	---	---	0.615	0.350	1.000	0.618	0.429	0.962	0.750	0.125	1.000	0.875	1.000
100	---	---	---	---	---	---	---	---	---	---	0.875	---	---	---
108	---	---	---	---	---	---	---	---	---	---	---	---	0.125	---
Ldh-1														
97	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	0.667	0.200	1.000	1.000	1.000	0.885
107	---	---	0.200	---	---	---	---	---	0.333	0.800	---	---	---	0.115
Ldh-2														
108	1.000	1.000	0.900	0.955	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
112	---	---	0.100	0.045	---	---	---	---	---	---	---	---	---	---
Hbdh														
105	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.933
120	---	---	---	---	---	---	---	---	---	---	---	---	---	0.067
Mdh-1														
90	---	---	---	0.020	---	---	0.038	0.077	0.024	0.050	---	---	---	---
100	1.000	1.000	1.000	0.980	1.000	1.000	0.962	0.923	0.9876	0.950	1.000	1.000	1.000	1.000
Mdh-2														
107	---	---	---	---	---	---	---	---	0.240	0.250	---	---	---	---
115	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.760	0.750	1.000	1.000	1.000	1.000
125	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Mdhp-1														
110	1.000	1.000	1.000	1.000	1.000	1.000	0.250	0.107	0.967	0.750	0.350	1.000	1.000	0.654
118	---	---	---	---	---	---	0.750	0.893	0.033	0.250	0.650	---	--	0.308
126	---	---	---	---	---	---	---	---	---	---	---	---	---	0.038
Mdhp-2														
94	0.300	0.900	1.000	1.000	1.000	1.000	0.806	1.000	0.717	1.000	0.500	0.300	0.300	0.692
103	0.700	0.100	---	---	---	---	0.194	---	0.283	---	0.500	0.700	0.700	0.308
Idh-1														
90	---	---	---	---	---	---	0.033	---	---	---	---	---	---	---
94	1.000	1.000	1.000	0.981	1.000	1.000	0.967	1.000	1.000	1.000	1.000	1.000	1.000	1.000
103	---	---	---	0.019	---	---	---	---	---	---	---	---	---	---
Idh-2														
97	---	---	---	---	---	---	0.033	---	---	---	0.625	0.600	0.250	0.778
100	1.000	1.000	1.000	1.000	0.950	1.000	1.000	1.000	1.000	1.000	0.375	0.400	0.750	0.222
110	---	---	---	---	0.050	---	0.033	---	---	---	---	---	---	---
6Pgdh														
87	0.300	0.353	0.550	0.400	0.650	0.792	0.778	0.333	0.250	0.800	0.875	0.100	0.312	0.692
95	---	---	---	---	---	---	0.083	---	---	---	---	---	---	
97	0.600	0.559	0.350	0.200	0.350	0.208	0.222	0.583	0.700	0.200	0.125	0.900	0.668	0.308
103	0.100	0.098	0.100	0.010	---	---	---	---	0.050	---	---	---	---	---
Gapdh														
93	1.000	1.000	1.000	0.542	1.000	1.000	0.929	0.875	1.000	1.000	1.000	1.000	1.000	1.000
96	---	---	---	0.458	---	---	---	---	---	---	---	---	---	---
105	---	..---..	---	---	---	---	0.071	0.125	---	---	---	---	---	---
Aat-1														
90	---	---	0.034	---	---	---	---	0.037	---	---	---	---	---	---
103	0.800	1.000	1.000	0.121	---	---	0.111	0.423	0.400	---	---	---	---	---
110	0.200	---	---	0.845	0.950	1.000	0.889	0.577	0.567	1.000	1.000	1.000	1.000	1.000
120	---	---	---	---	0.050	---	---	---	---	---	---	---	---	---
Aat-2														
88	1.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
105	---	---	---	0.017	---	---	---	---	---	---	---	---	---	---
Ck														
90	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.400	1.000	1.000	1.000	1.000
100	---	---	---	---	---	---	---	---	---	0.600	--	---	---	---

Following

Table S1: Allozyme allele frequencies

	Sample													
Locus	SRI	STA	SGA	STO	SSL	STR	SSG	STC	STE	STN	SLU	SPE	SMB	SAS
Allele	16	17	18	19	20	21	23	24	25	26	27	28	29	30
Pgm-1														
92	0.300	0.235	0.400	---	---	---	---	---	0.033	---	---	---	---	---
102	0.700	0.765	0.600	1.000	1.000	1.000	1.000	1.000	0.967	1.000	1.000	1.000	1.000	1.000
Pgm-2														
86	---	---	---	0.020	---	---	---	---	---	---	---	---	---	---
92	1.000	1.000	1.000	0.980	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Est-4														
95	---	---	---	---	---	---	0.333	0.071	---	---	---	---	---	---
100	---	---	---	---	---	---	---	---	0.115	---	---	---	---	---
105	0.700	0.917	0.900	0.457	---	---	0.167	0.143	0.270	0.200	0.500	0.500	---	0.462
110	0.300	0.083	0.100	0.543	1.000	1.000	0.500	0.786	0.615	0.800	0.500	0.500	1.000	0.538
Ap														
95	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pep-D														
90	1.000	0.833	1.000	1.000	1.000	1.000	0.417	0.667	1.000	1.000	1.000	0.667	0.750	0.400
96	---	0.167	---	---	---	---	0.583	0.333	---	---	---	0.333	0.250	0.600
Ada-1														
85	---	---	---	---	---	---	---	---	---	---	---	---	---	---
98	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.077
C	---	---	---	---	---	---	---	---	---	---	---	---	---	0.923
Ada-2														
100	---	---	---	---	---	---	---	---	---	---	---	---	---	---
103	0.900	1.000	1.000	0.172	0.222	0.208	0.389	0.667	1.000	---	1.000	1.000	1.000	1.000
106	---	---	---	---	---	---	---	---	---	---	---	---	---	---
110	0.100	---	---	0.828	0.778	0.792	0.611	0.333	---	---	---	---	---	---
Ca-2														
90	1.000	1.000	1.000	0.958	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
100	---	---	---	0.042	---	---	---	---	---	---	---	---	---	---
Ca-3														
82	---	---	---	---	---	---	---	---	---	---	---	---	0.038	---
90	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.962	1.000
Gpi														
93	---	---	---	---	---	---	---	---	---	---	0.050	---	---	---
9	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.950	1.000	1.000	1.000

Figure S1. Mitochondrial chronogram for *H. strinatii*. Maximum clade credibility tree obtained from the mitochondrial dataset. Red bars at nodes represent 95% Highest Posterior Densities of node ages. The split node between *H. strinatii* and Eastern Sardinian species (*H. supramontis* and *H. imperialis*) was constrained at 5.33 ma, which is the end of the Messinian salinity crisis.



3. THIRTY YEARS OF ARTIFICIAL SYNTOPY BETWEEN *HYDROMANTES ITALICUS* AND *H. AMBROSII AMBROSII* 4. (AMPHIBIA, PLETHODONTIDAE)

Cimmaruta R., Forti G., Lucente D., Nascetti G.

Abstract

Thirty years ago, in 1983, an experiment of artificial syntopy put together two allopatric taxa of mainland European plethodontids: *Hydromantes ambrosii ambrosii* and *H. italicus*. An equal number of specimens of both species were released in a cave with a suitable environment but located outside the range of the genus. The aim was to test the effectiveness of the reproductive isolating mechanisms of these two moderately divergent taxa and, in the case hybridization would have occurred, to analyse the extent and mechanisms of introgressive hybridization while in progress. Previous data collected between 1996 and 1999 showed that *H. italicus* and *H. a. ambrosii* were hybridizing and that their hybrids were viable and fertile enough to produce backcrosses. The data presented here, based on allozymes and restriction enzymes on a fragment of the mitochondrial Cytochrome-b gene, showed that introgressive hybridization is still ongoing. However, the gene exchange between the two taxa is restricted since most of the specimens scored were pure *H. a. ambrosii*, the percentage of hybrid/recombinant specimens was quite low and a strong deficiency of heterozygote genotypes was recorded. The results presented showed that this long term experiment assisted in providing insights into the patterns and mechanisms underlying hybridization and introgression, showing the spreading of a foreign mtDNA (*H. italicus*) into the gene pool of another species (*H. a. ambrosii*) despite the fact that the nuclear genomes remain substantially differentiated.

Keywords: allozymes, artificial syntopy, Cytochrome-b, *Hydromantes ambrosii ambrosii*, *H. italicus*, introgressive hybridization, RFLPs.

3.1 INTRODUCTION

Introgressive hybridization, defined as the gene exchange among differentiated species, is an evolutionary process whose relevance in introducing genetic and phenotypic novelty into species has been acknowledged at different times in plants and animals. In plants, hybridization has been recognised as a major force since the second half of the 20th century, and the bulk of evidences has become increasing with the passing of the decades (Anderson, 1949; Anderson and Stebbins, 1954; Grant, 1981; Baack and Rieseberg, 2007). In animal species, recent data increasingly support the hybridization process as much more widespread and evolutionarily relevant than previously thought, thanks to the outcomes of molecular investigation (Arnold, 1997; Gardner, 1997; Seehausen, 2004; Mallet, 2005, 2007). Moreover, some animal groups are particularly prone to hybridize, with rates equal to or even exceeding the 25% estimated for vascular plants. These include British ducks and game birds, tits, birds of paradise, American warblers and passion flower butterflies (Mallet, 2005).

The outcome of hybridization may be as different as the progressive and complete merging of the taxa involved, up to the formation of new hybrid species through either polyploidy or recombinational speciation, depending on both endogenous and exogenous factors (Grant, 1981; Rieseberg, 1997;Willis et al., 2006; Nolte and Tautz, 2010). As a consequence, the study of hybridization mechanisms both in natural and controlled conditions is becoming increasingly relevant thus allowing a better understanding of the extant patterns of diversity and adaptation (Mallet, 2007).

Introgressive hybridization is quite frequent in plethodontid salamanders, often subdivided in genetically closed and hybridizing species complexes. As an example, differential introgression is widespread in *Plethodon*,most likely due to the rapid radiation undergone by this genus in the southern Appalachians, from which originated a high number of moderately divergent species generating many hybrid zones whose features depend on selective regimes, behaviour, and habitat characteristics (Highton and Peabody, 2000;Wiens, Engstrom and Chippindale, 2006; Chatfield et al., 2010). This is the case of the species belonging to *P. glutinosus* complex, which also exchange genes with *P. shermani*, a species belonging to the *P. jordani* complex (Weisrock, Kozak and Larson, 2005; Wiens, Engstrom and Chippindale, 2006). Another widely studied case is that of *Ensatina eschscholtzii* complex, a ring species whose seven subspecies replace each other in intergradations zones and whose terminal taxa are still exchanging genes in contact zones, showing different patterns according to ecological conditions, local selection and outcomes of reinforcement (Wake, 2006; Devitt, Baird and Moritz, 2011).

The extent and relevance of hybridization in the European plethodontid salamanders of the genus *Hydromantes* has been analysed marginally so far. The distribution of the eight known species is mainly parapatric but no overlap nor hybrid zones have been reported between the five species inhabiting the island of Sardinia (Chiari et al., 2012), and a single hybrid zone has been found in the Apuan Alps (northern Tuscany) between *H. italicus*, the southernmost mainland species, and *H. ambrosii bianchii* (Nascetti et al., 1996; Ruggi et al., 2005). The other mainland taxa do not overlap and show a differentiation comparable to that reported between the hybridizing *H. italicus* and *H. a. bianchii* (Nascetti et al., 1996). It is therefore difficult to assess if *Hydromantes* taxa are reproductively isolated and if introgression has had a role in moulding their gene pool.

In order to weigh up the effective incidence of reproductive isolation among species vs. continuity between populations and species, it is relevant to understand both patterns of diversity and speciation

mechanisms in relation to the environment (Mallet, 2008). To this end, experiments of artificial syntopy are a powerful tool either to complement the data obtained from naturally hybridizing taxa in different environmental conditions or to artificially create a syntopy between parapatric or allopatric taxa with the aim to study the outcome (Cimmaruta et al., 1999; Parris, Semlitsch and Sage, 1999; Parris, 2000; Rull et al., 2012). Here we report the results obtained when putting two allopatric taxa of mainland European plethodontids: *H. a. ambrosii* and *H. italicus* in syntopy.

The experimental syntopy between *H. a. ambrosii* and *H. italicus* was carried out outside the range of *Hydromantes* species, to avoid accidental mixing and/or hybridization of foreign specimens into an alloctonous range. The site used for the experiment was the limestone cave "Buca della Nebbia", located near Monteriggioni, in Tuscany (central Italy). The release experiment started in April 1983 by releasing in the cave 30 specimens of *H. a. ambrosii* (16 males and 14 females) from the environs of La Spezia and 30 specimens of *H. italicus* (13 males, 14 females, 3 subadults) from the environs of Firenze. Both species survived in the cave and in 1996 newborns were observed for the first time. Between 1996 and 1999, a number of specimens were recovered from the cave and identified by means of multilocus electrophoresis carried out on tail tip tissue. F1 hybrids and adult backcrosses F1 × *H. italicus* were found, together with specimens of *H. a. ambrosii* and two individuals of *H. italicus* (Forti et al., 2005).

In October 2012 a new survey was carried out, revealing that the cave is still inhabited. The genotypes of the recovered specimens were assessed by means of allozyme electrophoresis and, for the first time, mitochondrial haplotypes were determined using restriction enzymes on a fragment of the Cytochrome-b gene. These data should provide insights into the extent and mechanisms of hybridization between *Hydromantes* closely related species.

3.2 MATERIALS AND METHODS

The survey at Buca della Nebbia (cadastral number 257 T/SI) was carried out on 18 October 2012. A total of 35 specimens were captured: 22 adults (recorded as A26 to A47) and 13 young or subadult salamanders (Y26 to Y38). A small part of the tail tip and a middle phalanx (the latter from adults only) were taken and immediately frozen in liquid nitrogen for allozymes and mtDNA analysis. The specimens were then re-released into the cave.

The tissue from the tail was used to carry out allozyme electrophoresis on 4 loci discriminating between *H. a. ambrosii* and *H. italicus*: Lactate dehydrogenase (E.C. number 1.1.1.28; *Ldh-1*, *Ldh-2*), Creatine kinase (E.C. number 2.7.3.2; *Ck*), Malate dehydrogenase (NADP+) (E.C. number 1.1.1.40; *Mdhp-2*). The partially discriminant locus 6-Phosphogluconate dehydrogenase (E.C. number 1.1.1.43; *6-Pgdh*) was also scored. The electrophoretic techniques are reported in detail in Lanza et al. (1995).

The phalanx tissue and the residue electrophoresis homogenate were used to extract total DNA according to CTAB/phenol-chlorophorm protocol slightly modified from Murray and Thompson (1980). PCR amplification of a 688 bp long fragment of the gene Cytochrome-b (Cyt-b) was carried on using the specifically designed primers HST-1f 5'-TTTATTGATTTACCAACCCCATCT-3' and DEB-1r

5'-CAGGGGTGAAGTTTTCTGGAT-3'. PCR protocol is as described by Ruggi et al. (2005) with minor modifications (Marusso et al., 2013).

The restriction fragment length polymorphisms (RFLPs) were analysed using the following endonuclease enzymes for the digestion of the amplified Cyt-b fragment: HaeIII, SspI, RsaI, MboI. Enzymatic digestions were performed in a total volume of 10 μl containing 7.5 μl of the PCR product, 1 μg of BSA and 5u of endonuclease. The digested DNA fragments were run on agarose 3% gel. The digestion patterns were identified by their comparison with a suitable DNA step ladder marker, each providing distinct fragment sets for *H. italicus* and *H. a. ambrosii*.

Allele frequencies and Hardy-Weinberg proportions were calculated using GenoDive 2.0b23 software (Meirmans and Van Tienderen, 2004; http://www.patrickmeirmans.com/software/).

A Bayesian approach using allele frequencies of multilocus genotypes was used to assign each individual to a genotypic class, as implemented in the software NewHybrids (Anderson and Thompson, 2002; http://ib.berkeley.edu/labs/slatkin/eriq/index.htm). The calculation was carried out following Anderson and Thompson recommendations: 105 iterations after a burn-in of 105 iterations. The genotypes of specimens from the same source populations used to carry out the experiment of artificial syntopy (from the environs of Florence and the environs of La Spezia) have been used as prior information, thus providing the allele frequencies of pure taxa. However, they were discarded from the calculation of the proportion of hybrids of each category in the sample from Buca della Nebbia (options z and s in NewHybrids, according to Anderson, 2003). NewHybrids assigns the genotypes to the six possible classes formed during two generations of crosses, i.e. pure *H. italicus* or *H. a. ambrosii*, F1 or F2 hybrids, Backcross $\times$ *H. italicus* or Backcross $\times$ *H. a. ambrosii*. This means that this software does not identify hybrids beyond the second generation, but specimens with $P < 0.95$ can be interpreted as later generation hybrids (Zha, Milne and Sun, 2008). In our case, the individuals were assigned to the genotypic class showing $P \geq 0.93$, since this was the lower posterior probability assigned to specimens from the source populations.

3.3 RESULTS

Thirty-one specimens out of the 35 analyzed for allozymes provided reliable results and their allele frequencies are reported in table 1, together with the allele frequencies of the source populations, recorded from samples taken during the same years of the release experiment (1980-85).

The allelic frequencies recorded at Buca della Nebbia showed that there was a prevalence of *H. a. ambrosii* alleles with percentages between 0.75 (*Mdhp-2*) and 0.84 (the other loci). The locus *6-Pgdh* was not completely discriminant between the two taxa, but the allele *6-Pgdh100* was recorded at 84% in the source population of *H. italicus* while it was at 35% in the source population from La Spezia (*H. a. ambrosii*), where the most frequent allele was *6-Pgdh110*, found at 65%. Also, pure *H. italicus* presented the allele *6-Pgdh103* at 11%, which has never been observed in *H. a. ambrosii*. The frequencies recorded in the sample from Buca della Nebbia showed a high percentage of *6-Pgdh110*, found at 84%, with *6-Pgdh100* at 16%; the allele *6-Pgdh103*

was not detected. The genotype frequencies revealed a highly significant deviation from Hardy-Weinberg proportions, showing a remarkable deficit of heterozygotes at all the five scored loci in the Buca della Nebbia population (table 1).

The genotypic assignment performed by NewHybrids assigned *H. italicus* from source population to the class pure *H. italicus* with $0.80 > P > 0.93$ (the lower value of 0.80 is due to the polymorphism showed by a single specimen at *6-Pgdh*) and *H. a. ambrosii* from the source population to the class pure *H. a. ambrosii* with $0.98 > P > 0.99$. In relation to the specimens from Buca della Nebbia, 24 were assigned to pure H. a. ambrosii (18 adults and 6 young specimens): 22 with $P \geq 0.98$ and another 2 with $P = 0.80$. Two young individuals were assigned to pure H. italicus class (Y26 and Y27) with $P \geq 0.90$. An adult specimen was classified as F2 (A38, P = 51%) and 4 specimens as backcrosses with H. a. ambrosii (A32, Y37, Y38) or *H. italicus* (Y30) (fig. 1).

The analysis of RFLP showed that most of the specimens analysed carried a *H. a. ambrosii* haplotype: 25 out of 31. It is worth noting that two specimens carrying H. a. ambrosii haplotypes showed pure H. italicus genotypes (Y26, Y27). For two others (A33, A40) the genotypes were assigned to pure *H. a. ambrosii* ($P = 0.80$) but the observed haplotypes were *H. italicus*. The remaining four *H. italicus* haplotypes belonged to specimens assigned to hybrid classes: A32, A38, Y30, Y37 (fig. 1).

3.4 DISCUSSION

The data reported showed that 30 years after it started, an experiment of artificial syntopy between the allopatric taxa *H. italicus* and *H. a. ambrosii* is still continuing. During this timespan the salamanders have continuously inhabited the cave and have been reproducing since 1996. The genetic analyses carried out using both allozymes and mtDNA showed that the genome of *H. a. ambrosii* is currently overrepresented with respect to the time of release, when half of the specimens belonged to each taxon (Forti et al., 2005). At present time 71% (22/31) of the individuals recovered were assigned to pure *H. a. ambrosii* and all of them were born in the cave, since the expected life expectancy of these salamanders is around 25 years (Lanza et al., 2005). At the same time, no pure *H. italicus* were observed, since the two young specimens carrying *H. italicus* alleles in homozygosis had a *H. a. ambrosii* mitochondrial haplotype. This suggests that the reproductive success of this species was failing, as already indicated by the data reported by Forti et al. (2005), showing only two *H. italicus* specimens out of the 55 analysed between 1996 and 1999. Five of the 31 specimens analysed (16%) were allocated to hybrid classes by the software NewHybrids (Anderson and Thompson, 2002), with a quite low probability. Three of them had the highest assignment probability as *H. a. ambrosii* backcrosses, with one of them having *H. a. ambrosii* haplotype while the other two had *H. italicus* haplotypes; another was classified as *H. italicus* backcross and exhibited *H. italicus* haplotype and the last one as F2, with H. italicus haplotype. Since NewHybrids is designed to work over the six classes representing the first two generations of hybridization (see Materials and methods), the highly mixed genotypes generated by a higher number of generations (recombinants) are scored with greater uncertainty.

This is why specimens with$P < 0.95$ are usually interpreted as later generation hybrids (Zha, Milne and Sun, 2008). In our data, the individuals assigned to hybrid classes showed an assignment probability between

51% and 80%, thus suggesting that they were recombinant genotypes. The data obtained from mtDNA confirmed this hypothesis, showing that there were four specimens assigned to a pure parental species (either *H. italicus* or *H. a. ambrosii*) that however carried the mitochondrial genome of the other, thus suggesting repeated crosses over the many generations occurring in the Buca della Nebbia population over the 16 years since reproduction was observed for the first time. Moreover, there were two specimens with assignment probability to *H. a. ambrosii* of 80% that showed *H. italicus* haplotypes, thus confirming that low assignment probabilities identify recombinant genotypes.

The ongoing mixing of recombinant specimens showed that complete isolation mechanisms do not exist between the taxa involved. The hybrids are viable and fertile to some extent, in agreement with the pattern of differentiation observed between *H. italicus* and *H. a. ambrosii*. Indeed, these taxa have a level of genetic differentiation comparable to that observed between *H. italicus* and *H. a. bianchii* (DNei 0.19 vs. 0.40) that, when meeting on the Apuan Alps, originate a hybrid zone characterized by a massive presence of recombinant genotypes (Nascetti et al., 1996; Lanza et al., 2005).

On the other hand, the introgressive hybridization observed seems neither free nor symmetric, and most of the observed alleles come from *H. a. ambrosii*. This observation has several possible explanations. The first is that *H. a. ambrosii* may be a better competitor than *H. italicus*, which would have been progressively displaced from the cave. The displaced species would have been disappeared after a few hybridization events leaving its genes still present in the Buca della Nebbia population. Another possibility is that introgression is driven mainly by endogenous factors, making *H. italicus* genome highly permeable to foreign alleles and hence prone to being swamped by *H. a. ambrosii* alleles. Finally, since relatively few specimens (30 *H. a. ambrosii* + 30 *H. italicus*) were introduced into the cave, the possibility exists that only a very small number of individuals survived and/or reproduced. This would have produced a shift of the allelic frequencies in the Buca della Nebbia population by means of a bottleneck event, with an overrepresentation by pure chance of one species in relation to the other, namely *H. a. ambrosii*. It is difficult to decide between these hypotheses, but some considerations can be made. Both species have been placed in a foreign environment and the cave Buca della Nebbia is a moderately effective retreat so that the specimens released do not experience highly constraining conditions. As a consequence, a possible advantage due to a pre-adaptation to local environmental conditions seems quite unlikely. A relevant role of endogenous driving forces is supported by the highly significant deficit of heterozygotes observed, suggesting that hybrid/ recombinant genotypes may have a lower fitness than parental specimens, especially *H. a. ambrosii*. This would have produced a progressive increasing of *H. a. ambrosii* genes in the population gene pool with the passing of time, a pattern confirmed by the fact that only 29% of the specimens recorded are recombinant genotypes, against 71% of *H. a. ambrosii*. Finally, a prominent function of genetic drift seems quite likely. Indeed, the surveys carried out between 1983 and 1996 showed a very small number of specimens present in the cave (with a mean of 4 ± 1.05 specimens recovered per survey; Forti et al., 2005), suggesting an abrupt bottleneck of the population. The picture changed when young salamanders were observed for the first time inMay 1996: the estimated population size at that time rose to 41 (±9.8). However, *H. italicus* was already rare, represented by two adults only and by none of the young specimens of the 55 individuals sampled (4%). Sixteen years later (2012), pure *H. italicus* have disappeared, showing that the two adults present in 1996 did not leave any pure progeny. It is worth noting that the source population of *H. italicus* presented 3 different alleles at the differentiated locus *6-Pgdh*: *6-Pgdh100*, *6-Pgdh103*, *6-Pgdh110*. The rarest

allele *6-Pgdh103* was not recovered, supporting the hypothesis that a founder effect and/or genetic drift, both of which are known to act strongly on rare alleles, moulded the genetic diversity of the population of "Buca della Nebbia".

The data presented showed that *H. italicus* and *H. a. ambrosii* do hybridize and that their hybrids and recombinants are viable and fertile. However, the gene exchange between the two taxa is restricted since most of the specimens scored were pure *H. a. ambrosii*, the overall number of hybrid/recombinant specimens was quite low and a strong lacking of heterozygote genotypes was recorded. In addition, the study of the hybrid individuals strongly suggests that the first crosses were asymmetric, involving *H. italicus* females and males *H. a. ambrosii*: only three specimens out of the 9 individuals carrying alleles of both species showed a *H. a. ambrosii* haplotype, in spite of the fact that the nuclear alleles were largely *ambrosii* for all but two. This picture provides interesting insights into the mechanisms of introgression, showing the spreading of a foreign mtDNA in to the gene pool of a species (in this case *H. a. ambrosii*) despite the fact that the nuclear genomes remain substantially differentiated and suggesting a role for endogenous factors. Finally, the results presented confirm that long term experiments assist in understanding patterns and mechanisms underlying hybridization and introgression.

3.5 ACKNOWLEDGEMENTS

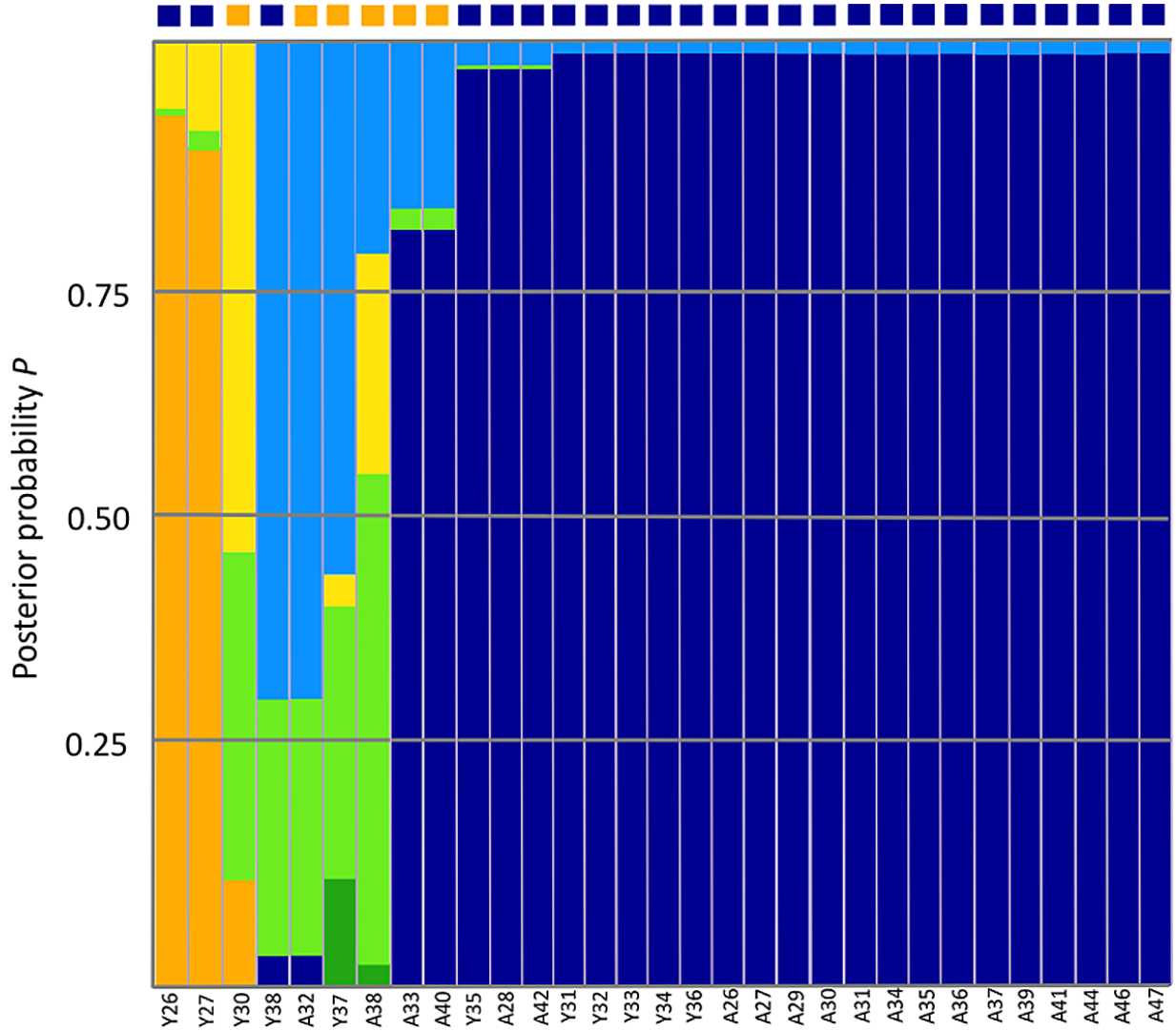
We wish to acknowledge Prof. Bettino Lanza, the pioneer of this release experiment, responsible for its planning and implementation. Of greater relevance, he introduced us to the wonderful subterranean world of *Hydromantes* which, after thirty years, still captivates us. We are indebted to Dr. Paola Arduino, whose assistance enabled us to score allozymes from an infinitesimal piece of tail tissue and to Bruno and Marco Fabiani for valuable help in collecting *Hydromantes*. Our thanks also to Sara Rosenman who kindly reviewed the English.

3.6 TABLES AND FIGURES

Table 1. The allelic frequencies at the 5 scored loci in the sample from Buca della Nebbia (NEB, artificial syntopy) and in the two populations used as sources of the released specimens (IFI, *H. italicus* and ASP, *H. a. ambrosii*). The significance of deviation from Hardy-Weinberg proportions is reported per locus per population (NS, Not Significant).

Locus	NEB	IFI	ASP
Ldh-1 85	0.84	–	1.00
Ldh-1100	0.16	1.00	–
	P <0.05		
Ldh-2100	0.16	1.00	–
Ldh-2108	0.84	–	1.00
	P <0.05		
Ck90	0.84	–	1.00
Ck100	0.16	1.00	–
	P <0.05		
Mdhp-296	0.75	–	1.00
Mdhp-2100	0.25	1.00	–
	P = 0.001		
6-Pgdh100	0.16	0.84	0.35
6-Pgdh103	–	0.11	–
6-Pgdh110	0.84	0.05	0.65
	P <0.05	NS	NS

Figure 1. Posterior probability distribution of the *Hydromantes* salamanders recovered from the cave Buca della Nebbia. The probability of genotypic assignment per class, the scored haplotypes (blue squares = *H. a. ambrosii*; yellow squares = *H. italicus*) and the specimen labels are reported. Dark yellow = *H. italicus*; dark blue = *H. a. ambrosii*; dark green = F1; light green = F2; light yellow = backcross × *H. italicus*; light blue = backcross × *H. a. ambrosii*.



4. DISCUSSIONE GENERALE

Quanto emerso nel presente lavoro, aggiunge ulteriore conferma al fatto che le specie caratterizzate da bassa vagilità, nonché da popolazioni piccole e frammentate, si configurano come sistemi biologici evolutivamente complessi, per i quali l'attuale assetto della struttura genetica è quasi sempre il risultato di una lunga storia di interazioni tra fattori di diversa natura. Districare la rete di queste strette interazioni e i fattori che più hanno influenzato i processi di isolamento, divergenza e di eventuale introgressione delle linee evolutive non è senza dubbio semplice. Tuttavia, specie così caratterizzate possono essere considerate dei veri e propri laboratori naturali grazie ai quali è possibile osservare le diverse forze evolutive in azione.

Le tre specie continentali europee del genere *Hydromantes*, scelte per questo lavoro, rispondono perfettamente a questi pattern generali, ai quali aggiungono la peculiare caratteristica delle abitudini troglofile.

I processi microevolutivi che interessano queste specie sono stati analizzati attraverso approcci differenti, ma i cui risultati, letti complessivamente, consentono un'interpretazione più ampia dei fenomeni indagati. Nello specifico, i risultati derivati dall'analisi filogeografica del geotritone di Strinati sono stati interpretati anche alla luce di quanto emerso dall'esperimento di sintopia artificiale tra *H. a. ambrosii* e *H. italicus*.

Tra i geotritoni italiani, *H. strinatii* è tra quelli che presentano i più elevati livelli di frammentazione geografica e genetica delle popolazioni e ciò è in buona parte da attribuire all'orografia e all'elevata eterogeneità ambientale che caratterizzano l'intero areale della specie, distribuita tra la Toscana settentrionale, la Liguria e le Alpi Liguri e Marittime. L'integrazione delle informazioni derivanti dai diversi marcatori utilizzati per l'analisi filogeografica suggerisce che, in questo pletodontide, sia la distribuzione geografica delle principali linee evolutive (ovvero i due cladi mitocondriali A e B), sia il loro differenziamento ed i loro livelli di variabilità genetica siano il risultato tanto di processi paleogeografici e paleoclimatici, quanto di processi di flusso genico, deriva genetica e lineage sorting incompleto.

Lo scenario emergente è quello di una tendenza delle popolazioni di *H. strinatii* a persistere in condizioni di forte isolamento, pur mantenendo le piccole dimensioni numeriche tipiche della specie. Ciò promuove un accumulo di diversità genetica in allopatria come testimoniato dall'elevato grado di differenziamento (a livello sia nucleare sia mitocondriale) riscontrato tra le popolazioni. In questo contesto la deriva sembra assumere un ruolo importante nel guidare i processi di diversificazione osservati. Infatti, la forte strutturazione geografica e la presenza di aplotipi per buona parte esclusivi di singoli siti che si osservano al mitocondriale è la firma riconoscibile dell'azione della deriva genetica, che facilita la fissazione di nuove mutazioni all'atto di fondazione di nuove popolazioni. Però, una volta che una popolazione si è stabilita nel nuovo habitat, la successiva comparsa di nuovi aplotipi, per mutazione o per rari eventi di dispersione, ha poca probabilità di fissarsi a causa di un effetto di "priorità del residente" o per effetto del "mutation surfing". Il primo si verifica quando, dopo un evento di fondazione, una popolazione cresce in dimensioni a partire da pochi individui e la divergenza delle varianti geniche che si sono stabilite è tale da opporre resistenza alla fissazione di eventuali nuove mutazioni che arrivano successivamente per flusso genico (Boileau et al., 1992). Il secondo effetto, invece, si verifica quando, durante un'evento di espansione, alleli inizialmente rari o nuove mutazioni vanno incontro ad un incremento della frequenza e della distribuzione spaziale e questo dipendentemente dalle pressioni selettive che agiscono sugli individui nel fronte di espansione (Traviset al., 2010).

Per quanto riguarda i loci allozimici, invece, l'azione della deriva genetica si è tradotta in una variazione in parte casuale delle frequenze alleliche attraverso il range della specie.

La casualità di questi processi microevolutivi, tende a confondere i reali rapporti filogenetici tra le popolazioni esaminate. A ciò si aggiunge il fatto che i marcatori allozimici, se da un lato sono generalmente informativi nel rivelare i pattern di differenziamento tra popolazioni, dall'altro possono subire, nel corso del tempo, un effetto di saturazione, rendendo ulteriormente complessa l'inferenza filogeografica. Tuttavia, l'integrazione delle informazioni derivanti dai due dataset molecolari, nonché la parziale concordanza tra questi ultimi, consentono di delineare complessivamente uno scenario evolutivo ben supportato e diversificato sia a livello geografico (con i gruppi di popolazioni ad est ed ovest dell'areale che presentano strutture e storie ben diverse) sia a livello di meccanismi evolutivi in azione.

I valori molto più elevati a tutti gli indici di variabilità (diversità nucleotidica, diversità aplotipica, numero di alleli privati, eterozigosità attesa e percentuale di loci polimorfici) che sono stati riscontrati nei gruppi di popolazioni occidentali (ovvero quelle appartenenti ai sub-cladi B1, B2 e B3 e comprese tra Francia e Liguria occidentale), come anche il consistente differenziamento tra questi emerso dai dati mitocondriali, sembrano favorire l'ipotesi che la zona geografica in cui ricadono rappresenti un'area di ancestralità, dalla quale si sarebbero originate le popolazioni appartenenti ai sub-cladi più orientali (A1 e A2). Infatti, benchè i pletodontidi siano notoriamente animali sedentari e dotati di una notevole filopatria, le ricerche filogeografiche condotte negli anni hanno messo in luce che questi anfibi sono stati capaci di andare incontro a significative espansioni del proprio areale e colonizzazioni di nuovi habitat. Ed è soprattutto sulla base di tali evidenze che è stata avanzata l'ipotesi che in queste specie possano verificarsi degli eventi di dispersione occasionale, dovuti al fatto che i pletodontidi, in generale, possiedono la capacità di disperdersi su lunghe distanze, ma che utilizzano questa abilità solo di rado (Cabe et al., 2007). Un'origine delle popolazioni più orientali a partire da quelle occidentali spiegherebbe perché gli individui appartenenti ai sub-cladi A1 e A2 condividano agli allozimi alcuni alleli tipici anche delle popolazioni appartenenti ai due sub-cladi dell'estremità più occidentale (B2 e B3), ovvero quelle delle Alpi Marittime francesi e di Tenarda. Due ipotesi sono state considerate al fine di spiegare tale pattern: una prevede un contatto secondario tra i due gruppi di popolazioni, l'altra un incomplete lineage sorting. Distinguere tra queste due possibilità non è sempre semplice, ma in linea generale la discordanza causata da un lineage sorting incompleto non tende a lasciare alcun pattern biogeografico prevedibile, quali la prossimità geografica di alleli condivisi tra popolazioni distinte che è invece tipico dei casi di introgressione (Funk e Omland, 2003; Toews e Brelsford, 2012). Sulla base di questo, la spiegazione più parsimoniosa sembra essere quella di un lineage sorting incompleto, un fenomeno per altro frequente per i marcatori nucleari rispetto a quelli mitocondriali: infatti il genoma mitocondriale, avendo una dimensione di popolazione effettiva pari ad un quarto di quella nucleare tende ad avere tempi di coalescenza inferiori e dunque a raggiungere prima la condizione di monofilia reciproca tra linee evolutive divergenti (Ballard e Withlock, 2004).

Un altro risultato indicativo dei processi evolutivi che hanno plasmato il pool genico di *H. strinatii* concerne gli individui di Tenarda e quelli delle Alpi Marittime francesi, i quali clusterizzano rispettivamente in due sub-cladi mitocondriali altamente divergenti tra loro e con livelli di strutturazione spiccati, ma che al contrario, sulla base dei loci allozimici, presentano un forte mescolamento dei pool genici senza alcuna chiara suddivisione in sottogruppi. L'elevata variabilità genetica emersa per queste popolazioni potrebbe essere imputata tanto ad un contatto secondario, quanto alla persistenza delle popolazioni di quest'area. Entrambe le ipotesi trovano qualche prova a sostegno, dato che l'andamento delle frequenze alleliche ad alcuni loci allozimici mostra dei clini, come atteso in caso di contatti secondari, ma l'eterogeneità ambientale e la storia paleogeografica dell'area sostengono la possibilità che le popolazioni delle Alpi Liguri e Francesi abbiano mantenuto la loro variabilità genetica grazie alla persistenza in questa zona di rifugio. Infatti, nonostante durante il Quaternario nella zona compresa tra le Alpi

Marittime e quelle Liguri si sia verificata la massima estensione meridionale del ghiaccio (Kropf et al., 2002), almeno durante l'ultimo massimo glaciale, nelle Alpi sud-occidentali la linea della neve era a circa 2000 metri sopra il livello del mare e questo supporterebbe l'ipotesi dell'esistenza di rifugi peri-glaciali lungo i bordi della catena alpina. A forte sostegno di questa ipotesi vi è un crescente accumulo di dati concordanti per diverse specie, che risultano a favore di uno scenario biogeografico ed evolutivo in base al quale queste specie sarebbero sopravvissute o in rifugi locali peri-glaciali presenti sui pendii meridionali o nei nunatak di questa zona (Christe et al., 2014; Schönswetter et al., 2005; Stefani et al., 2012). All'interno di questi rifugi locali multipli le specie si sarebbero mantenute in una condizione di forte frammentazione delle popolazioni, ma sarebbero comunque sopravvissute ed è proprio a partire da qui che si sarebbero verificate le successive espansioni post-glaciali. L'area delle Alpi Liguri e Marittime è quindi vista come un'area di rifugio glaciale, in cui le popolazioni hanno avuto la possibilità di persistere, continuando ad accumulare variabilità genetica. La struttura genetica del geotritone di Strinati nell'area delle Alpi Liguri e Marittime conferma questo quadro, con il dna mitocondriale che ha conservato le tracce storiche degli eventi pregressi di frammentazione e isolamento, manifestati sotto forma di una forte strutturazione genetica delle popolazioni e di elevati livelli di differenziamento anche tra popolazioni geograficamente vicine (quali quelle francesi). Questo stesso pattern è stato riscontrato anche in altre altre specie dell'area (Engelhardt et al., 2011; Garnier et al., 2004; Kropf et al., 2002). D'altro canto, i risultati delle analisi allozimiche (in particolare quelli relativi all'assegnazione statistica dei genotipi a popolazioni distinte) dimostrano come questo isolamento non sia stato assoluto e che episodi di contatto secondario si sono verificati tra le popolazioni delle Alpi Marittime francesi e quella di Tenarda, plausibilmente fornendo un contribuito importante al generale mantenimento dell'elevata variabilità complessiva di quest'area. A sostegno di questa ipotesi, altre specie residenti nell'area mostrano una elevata diversità che sembra essersi mantenuta grazie a migrazioni "up and down" a scala locale, lungo i pendii dei rilievi, in risposta alle oscillazioni del clima (Kropf et al., 2002). Quanto emerso dall'analisi filogeografica del geotritone di Strinati è assolutamente concordante con le evidenze accumulate nella letteratura degli ultimi anni per specie con popolazioni ricadenti tra le Alpi Marittime e Liguri e contribuisce a dare ulteriore conferma che quest'area geografica abbia non solo svolto un importante ruolo di rifugio glaciale, ma che si configura anche come un vero e proprio hotspot di biodiversità.

Il pattern di diversità che *H. strinatii* presenta nell'area delle Alpi Marittime e Liguri è risultato conforme alle attese in base al modello del "micro-rifugio" (Mosblach et al., 2011), secondo il quale specie associate a micro-rifugi, come possono essere le grotte, riescono a mantenere popolazioni persistenti anche in condizioni climatiche avverse, mantenendo così la variabilità genetica ancestrale a livello intrapopolazionale, invece di perderla durante l'habitat shift collegato alle variazioni climatiche. Il pattern che ci si aspetta in questi casi qundi è di una forte strutturazione geografica associata ad elevata variabilità a livello nucleotidico ed aplotipico, unitamente alla mancanza di tracce di espansione recente, proprio come nelle popolazioni occidentali di *H. strinatii*. L'alta variabilità genetica può essere mantenuta anche grazie a episodi di flusso genico tra popolazioni di micro-rifugi diversi. L'aver trovato evidenza di un mescolamento dei pool genici nei dati nucleari senza alcun relativo riscontro in quelli mitocondriali, ci permette di non escludere l'ipotesi che i contatti siano stati mediati da una dispersione a carico del sesso maschile. Prove a supporto di ciò derivano da alcuni studi presenti in letteratura, secondo i quali il sesso che si muove di più è sempre quello maschile, a prescindere dalla scala temporale alla quale vengono effettuati gli studi (1 o 5 anni) (Liebgold et al., 2011; Pastorelli et al. 2002; Staub et al., 1995). Inoltre, Ruggi et al. (2005), studiando una zona ibrida tra *H. a. bianchii* e *H. italicus*, sia con marcatori nucleari sia mitocondriali, hanno evidenziato che nella zona di contatto erano presenti solo genotipi ricombinanti mentre non vi era condivisione di aplotipi dell'una e dell'altra specie nelle varie località in esame, con l'eccezione di un singolo sito localizzato al

centro della zona ibrida; un risultato che spinge fortemente a ipotizzare una maggiore vagilità dei maschi. Al contrario, l'esperimento artificiale descritto nel presente lavoro ha mostrato come, quando messe in condizioni "obbligate" di sintopia, l'introgressione tra *H. ambrosii* e *H. italicus* riguarda geni sia nucleari sia mitocondriali. Questo insieme di dati conferma che in *Hydromantes*, come in altri pletodontidi, fenomeni di ibridazione ed introgressione non sono ostacolati né a livello prezigotico né a livello post-zigotico, anche fra taxa geneticamente differenziati. Gli stessi dati però evidenziano che i meccanismi di ibridazione ed introgressione hanno modalità diverse in natura, rispetto a quanto osservato in situazioni di artificialità: nel primo caso si riscontra una discrepanza tra marcatori mitocondriali e nucleari che non si verifica nella sintopia artificiale, a dimostrazione del fatto che il contributo dei due sessi al flusso genico non è paritario. Inoltre nei casi studiati su popolazioni naturali (questa tesi, Ruggi et al., 2005) l'introgressione non è nè simmetrica nè equivalente a tutti i loci differenziati.

Altro aspetto che emerge dai nostri dati è che all'interno di *H. strinatii*, i livelli di divergenza osservati tra le singole linee filogeografiche sulla base dei dati mitocondriali sono risultati paragonabili a quelli stimati tra le specie continentali. Nel nostro caso, le stime del p-distance avevano un range compreso tra 0.8% e 5.2%. Già in un precedente lavoro di Forti et al., 1997 invece, utilizzando marcatori allozimici, gli Autori avevano riscontrato all'interno di *H. ambrosii* distanze di Nei (1972) simili (0.001-0.218) a quelle tra *H. ambrosii* e *H. strinatii* (0.23-0.48). Inoltre, in un lavoro sulla filogeografia dei geotritoni sardi, in cui sono stati inclusi anche esemplari delle tre specie continentali, gli Autori avevano osservato che, sulla base dei dati mitocondriali, le distanze genetiche tra le specie sarde erano generalmente più elevate di quelle stimate tra le specie continentali: il valore di p-distance medio tra *H. ambrosii* e *H. italicus* era risultato di 5.1% (con un range compreso tra 4.5% e 5.9%) (Chiari et al. 2012). Considerando le sole distanze genetiche, i livelli di differenziamento tra le specie continentali non sono tali da lasciar ipotizzare che l'isolamento riproduttivo completo sia stato raggiunto. A conferma di ciò, l'ibridazione introgressiva è stata provata tra *H. italicus* e *H. ambrosii*, sia in condizioni naturali sia artificiali.

L'esperimento di sintopia artificiale, di cui ci siamo occupati in questo lavoro, ha fornito risultati interessanti sotto diversi profili.

La popolazione attuale di Buca della Nebbia presenta un evidente squilibrio nella sua composizione relativamente alla composizione che, trent'anni fa, caratterizzava la popolazione di partenza e che vedeva le due specie equirappresentate (Forti et al., 2005). Ad oggi infatti, *H. italicus* sembra essersi del tutto estinto all'interno del sito, dal momento che nessuno degli individui da noi campionati è risultato corrispondere geneticamente a *H. italicus* puro. Tra le ipotesi prese in considerazione, quella più plausibile fa perno su un potenziale ruolo significativo della deriva genetica. Infatti, già nelle fasi iniziali dall'avvio dell'esperimento si era verificata una drastica riduzione della dimensione della popolazione, poiché solo pochi individui sono stati campionati nei primi tredici anni di sperimentazione. Poiché un effetto collo di bottiglia ha, come è noto, la conseguenza di ridurre le dimensioni effettive di una popolazione ed il livello complessivo di diversità genetica, questo spiegherebbe il cambiamento delle frequenze alleliche osservato, a tutti i loci analizzati, rispetto alle frequenze caratterizzanti le popolazioni di origine degli individui utilizzati nell'esperimento. Questo slittamento delle frequenze alleliche, si è mostrato particolarmente significativo per il locus 6Pgdh. Quest'ultimo presenta tre forme alleliche tra cui quella più rara, esclusiva di *H. italicus*, non è stata trovata in nessuno degli individui ricombinanti. Questo perché quanto più severo è il collo di bottiglia, tanto maggiore sarà la perdita iniziale di alleli e, in particolar modo, quella di alleli rari (Freeland e Kirk, 2011; Futuyma, 2008).

Tuttavia, come dimostrato, nonostante *H. italicus* si sia probabilmente estinto nel sito, circa un terzo degli individui campionati erano ricombinanti delle due specie parentali e, tra questi, la maggior parte presentava un aplotipo mitocondriale *H. italicus*. Da ciò si evincono due aspetti interessanti. Il primo è che l'ibridazione tra le due

specie porta alla formazione di una progenie ibrida vitale e fertile che continua a riprodursi nel corso di generazioni successive. In secondo luogo, il fatto che la maggior parte dei ricombinanti abbia un aplotipo *H. italicus* supporterebbe l'ipotesi per la quale l'introgressione mitocondriale tra i due taxa in questione sia un'introgressione asimmetrica, un fenomeno piuttosto frequente in natura (Mao et al., 2013; Melo-Ferreira et al., 2005; Toews and Brelsford, 2012; Zielinski et al., 2013), che nel caso specifico risulta sbilanciata verso una tendenza all'incrocio tra maschi *H. ambrosii* e femmine *H. italicus*. Tra i geotritoni italiani, un pattern analogo a quello di Buca della Nebbia è quello emerso dall'analisi di una zona ibrida, localizzata sulle Alpi Apuane, tra *H. italicus* e la sottospecie *H. a. bianchii*. In questo caso specifico, l'impiego dei marcatori nucleari allozimici ha messo in luce che in questa zona di contatto si verifica un'introgressione fortemente asimmetrica tra i due taxa, determinata da una diffusione di alleli tipici di *H. a. bianchii* nel pool genico di *H.italicus* molto più massiccia e geograficamente estesa di quanto non avvenga al contrario (Forti et al., 1997; Lanza et al., 2005; Nascetti et al., 1996; Ruggi et al., 2005).

L'ibridazione, nei Pletodontidi, è un fenomeno diffuso e da sempre ha rappresentato una sfida nei confronti del tentativo di delineare l'indipendenza effettiva delle linee evolutive (Wake, 2006). In generale, in questo gruppo, la divergenza genetica minima tra due cladi sopra la quale si verifica una sostanziale riduzione del flusso genico può essere piuttosto elevata, il che giustifica la frequenza con cui zone di contatto secondario sono state riscontrate in natura (Jockusch e Wake, 2002). Ciò non toglie che l'intensità di scambio genico tra popolazioni precedentemente isolate e l'estensione dell'ibridazione stessa dipendano, oltre che dal grado di isolamento riproduttivo raggiunto, anche da altri fattori, quali le proprietà adattative ed ecologiche delle specie parentali divergenti e la fitness della progenie ibrida (Barton, 2001; Grant e Grant, 2008). Tuttavia, la letteratura ci dimostra che anche un flusso genico limitato può essere sufficiente per l'instaurarsi di un'introgressione. Ad esempio *Plethodon shermani*, specie nordamericana appartenente al complesso di *P. jordani*, presenta una lunga storia di eventi di contatti secondari, seguiti da introgressione, con le specie del complesso di *P. glutinosus*, in tutti e quattro i distretti montuosi su cui la specie è presente. Tali eventi hanno avuto luogo in momenti diversi nel corso dell'evoluzione di questa specie, ma sono rimasti per lo più limitati ai confini tra i range dei taxa interessati. Pur non determinando una fusione completa tra i pool genici delle specie interagenti, questi eventi di introgressione hanno intaccato almeno parzialmente l'integrità genetica delle specie coinvolte.

In quest'ottica, l'esperimento di sintopia tra *H. a. ambrosii* e *H. italicus* ha messo indubbiamente in discussione il raggiungimento di una totale indipendenza evolutiva per questi due taxa, mostrando come l'isolamento riproduttivo sia tutt'altro che completo. Nonostante in natura non siano mai state trovate zone di contatto tra questi due taxa, ciò non toglie che episodi storici di flusso genico non possano essersi verificati. Infatti, come accennato in precedenza, le popolazioni naturali di *H. a. ambrosii* presentano alleli tipici di *H. italicus*. L'evidente assenza di un isolamento riproduttivo completo tra i due taxa, supporterebbe a questo punto l'ipotesi che tali alleli possano essere la testimonianza di una passata introgressione che potrebbe aver contribuito a determinare l'attuale variabilità genetica del geotritone di Ambrosi.

Alla luce di quanto fin'ora detto, si evince che i risultati ottenuti nel corso del presente lavoro aggiungono un tassello alla conoscenza dei fenomeni evolutivi che regolano e consentono il mantenimento della struttura genetica complessiva di specie caratterizzate da profili ecologici ed etologici peculiari, quali appunto quelli delle specie indagate. Queste peculiarità, ovvero la sedentarietà, la frammentazione delle popolazioni e le piccole dimensioni di queste ultime, si traducono in pattern evolutivi almeno parzialmente generalizzabili. Inoltre, l'antichità delle specie studiate offre testimonianza del fatto che la diversità genetica complessiva si è mantenuta fino ai nostri giorni grazie ad uno stretto gioco di interazioni tra forze microevolutive distinte che agiscono soprattutto a scala locale, in modo particolare sotto forma di deriva genetica e flusso genico. L'identificazione di queste forze evolutive si rivela

fondamentale lì dove il fine ultimo è quello di comprendere quali processi preservare nel tempo per assicurare il mantenimento del potenziale evolutivo di una specie.

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RINGRAZIAMENTI

Ho sempre pensato che nulla ci renda più vivi della continua curiosità di conoscere e di scoprire. Ed è la ragione per la quale credo che avere l'opportunità di fare ricerca scientifica sia un'occasione unica prima di tutto per se stessi. E' per questo che, alla fine di questi tre anni, il mio grazie più sentito va a coloro che mi hanno dato l'opportunità di intraprendere concretamente questa esperienza di dottorato: prima fra tutti la mia tutor, Roberta Cimmaruta, nella quale ho sempre trovato disponibilità ed entusiasmo; grazie anche al prof. Giuseppe Nascetti per aver contribuito a rendere possibile questo percorso.

Ringrazio il Dott. Daniele Canestrelli per tutti i consigli sempre preziosi.

Grazie ai miei colleghi della "Stanza Dottorandi" che, in modi diversi, mi hanno accompagnata in questi tre intensi anni. E, tra questi, un ringraziamento particolare va a Michela Paoletti per avermi pazientemente istruita nelle prime fasi di laboratorio. Ma non posso non includere in questa pagina anche Fabrizio Scialanca, Silvia Cecchetti e Paola Arduino per tutta la gentilezza e disponibilità che ho sempre trovato in loro.

Infine, un grazie speciale è dedicato ai miei genitori, a mia sorella, mio nipote e Simone, i miei punti fermi, al di là di ogni evento e senza i quali nessun percorso o traguardo avrebbero significato.