Megan L. Porter – Subterranean biogeography: what have we learned from molecular techniques? *Journal of Cave and Karst Studies,* v. 69, no. 1, p. 179–186.

SUBTERRANEAN BIOGEOGRAPHY: WHAT HAVE WE LEARNED FROM MOLECULAR TECHNIQUES?

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Abstract: Subterranean faunas have unique distributional attributes, including relatively small ranges and high levels of endemism. Two general models have been proposed to account for these distributional patterns–vicariance, the isolation of populations due to geographic barriers, and dispersal, an organism's ability to move to and colonize new habitats. The debate over the relative importance of each of these models in subterranean systems is ongoing. More recently, biogeographical studies of subterranean fauna using molecular methods have provided new perspectives into the distributional patterns of hypogean fauna, reinvigorating the vicariance versus dispersal debate. This review focuses on the application of molecular techniques to the study of subterranean biogeography, and particularly the contribution of molecular methods in estimating dispersal ability and divergence times. So far, molecular studies of subterranean biogeography have found evidence for the common occurrence of multiple independent colonizations of the subterranean habitat in cave-adapted species, have emphasized the importance of the genetic structure of the ancestral surface populations in determining the genetic structure of subsequent hypogean forms, and have stressed the importance of vicariance or a mixed model including both vicariant and dispersal events.

INTRODUCTION

Cave-adapted fauna have intrigued scientists for centuries. Part of this fascination has been focused on understanding the unique geographic distribution patterns over space and time (i.e., biogeography) of subterranean organisms. However, the unique suite of regressive (eye and pigment loss) and progressive (appendage elongation, enhanced non-visual sensory modes) traits termed troglomorphy (Christiansen, 1962) characterizing cavernicoles often hinder distributional studies because the highly convergent form can obscure taxonomic relationships among cave-adapted species and among closely related cave and surface species. Compared to surface species, cave-adapted faunas generally have small geographic ranges and high levels of endemism at all scales of measurement, making their biogeography distinct (Culver and Holsinger, 1992; Gibert and Deharveng, 2002; Christman et al., 2005). There are numerous records of single cave endemics in both terrestrial (troglobionts) and aquatic (stygobionts) cave-adapted species (Paquin and Hedin, 2004; Christman et al., 2005). These distinctive geographic patterns have lead to investigations of how, why, and when species colonize, adapt, and persist in subterranean environments. In general, understanding the biogeography of cave-adapted fauna offers insights not only into the evolution of the troglomorphic form, but also into the formation and persistence of subterranean faunas, providing important information relative to cave conservation and management issues.

There has been a long running debate regarding the mechanisms responsible for the distribution of cave-adapted fauna, beginning as early as the late 1800s (Packard, 1888). The crux of the debate has been over the relative roles of different biogeographic models, particularly dispersal (an organism's ability to move to and to colonize new habitats) and vicariance (isolation of populations due to geographic barriers). Over the years, various studies have supported one model or the other (see Culver et al., 2007 for a brief historical review). However, it has recently been recognized that subterranean faunal distributions are more clearly explained by a combination of both vicariance and dispersal events, with many reflecting processes occurring in ancestral surface populations before the invasion of the subsurface (Christiansen and Culver, 1987; Verovnik et al., 2004; Buhay and Crandall, 2005; Lefébure et al., 2006). With respect to the classic debate, subterranean distribution patterns are likely the result of complex processes both internal (e.g., dispersal capabilities) and external (e.g., vicariant events, habitat connectivity) to the species of interest. Therefore, rather than investigating biogeographical patterns in terms of one mechanism versus another, it has become more important to understand the combination of factors involved in creating current distribution patterns, including dispersal ability, potential vicariant events, and rates of evolution and extinction (Holsinger, 2005; Culver et al., 2007).

Given that there are ecological disparities controlling the distributional differences between troglobiotic and stygobiotic species (e.g., modes of colonization, rates of migration and extinction, types of geographic barriers), considerations in subterranean biogeography first include understanding the role of habitat on these factors (Holsinger, 2005). Subterranean aquatic environments are

generally connected over wider areas (due to hydrology) compared to the connectivity of karstic terrestrial habitats. Hydrologic connectivity provides stygofauna greater dispersal potential and, therefore, generally larger distributional ranges (Culver et al., 2007). Furthermore, the relative contribution of dispersal versus vicariance is dependent on factors such as the scale of investigation, ranging from faunal distributions under individual rocks, within cave stream riffles, in cave stream segments from a single system, within cave and karst basins of a single river drainage, from cave systems in different drainages, to regional and continental patterns (Culver and Fong, 1994). Investigating these diverse geographical and geological scales produces distributional patterns corresponding to differences in time and dominant processes, with large scale patterns (cave systems, regions, continents) occurring over geological / evolutionary timescales being strongly affected by vicariant and dispersal events, and distributions within cave systems occurring in ecological timescales with influences from processes such as competition, predation, mutualism, and migration.

As subterranean biogeographers begin to assess the relative roles of dispersal and vicariance in subterranean faunal distributions, molecular techniques, involving the characterization of genetic material like DNA, RNA, and proteins, have become an increasingly powerful tool, complementing the significant amounts of taxonomic and biogeographic research devoted to searching cave and karst systems for animals. The main goal of this overview is to explore the contributions of molecular data to our understanding of subterranean biogeography. I will discuss how recent molecular methods have provided the analytical tools to estimate phylogenetic relationships, population parameters (e.g., migration rates, population structure), and divergence times essential for gaining deeper insights into the colonization, persistence, and adaptation of fauna in subterranean settings. Molecular perspectives are also presented on several different scales, including populations versus species and karst basins versus continental distributions.

THE MOLECULAR PERSPECTIVE

Although classical genetics, where individuals from different populations are crossed to examine the heritability of particular traits, have a long history in biospeleology (Breder, 1943; Sadoglu, 1956), molecular techniques aimed at investigating the genetic variability of cave populations only began in the 1970s with the development of the first major molecular markers, allozymes (protein variants) (Avise and Selander, 1972; Carmody et al., 1972; Hetrick and Gooch, 1973; Laing et al., 1976; Cockley et al., 1977; Turanchick and Kane, 1979; Sbordoni et al., 1979). As the available number of molecular markers increased and the associated analyses became more sensitive and refined, investigations of subterranean biogeography from a genetic perspective became feasible (see Sbordoni et al., 2000 for review).

Currently, molecular studies using mitochondrial gene sequences to investigate population and species level questions are common, including the genes for 12S and 16S rRNA, cytochrome oxidase I, cytochrome B, and NADH dehydrogenase. Nuclear genes (e.g., 28S rRNA) have been less commonly used, and are generally more suitable for higher-level (among species, genera, families) phylogenetic studies. At the level of populations, genetic analyses utilizing molecular data, such as microsatellites (a sequence of DNA containing tandemly repeated units, where the number of repeats varies within and among populations) and DNA sequences, now allow for a vast range of parameters to be estimated and assessed for a particular species. These parameters include estimates of the number of genetic populations, migration rates (i.e., levels of gene flow), and effective population sizes (N_e is a measure of genetic diversity, calculated as the size of a hypothetical population where all of the adults contribute gametes to the next generation; N_e is usually smaller than the actual number of individuals in a population) (see Pearse and Crandall, 2004 for a review of recent advances in population genetics). At higher taxonomic levels (species and genera), molecular markers offer large numbers of characters to be used in phylogenetic (evolutionary) methods, increasing the sensitivity and resolution of the analyses. The following sections describe specific areas of investigation where, in coordination with the strong foundations of traditional biogeographic studies, molecular techniques have the potential to substantially increase our understanding of subterranean biogeography.

DISTRIBUTIONS OF CAVE ADAPTED SPECIES

One of the foundations of biogeographic studies is a solid understanding of the distribution of the species of interest, which can be difficult for cave-adapted species for several reasons. First, cave-adapted faunas are characterized by a suite of unique morphological (loss of eyes and pigmentation, elongation of appendages, hypertrophy of non-optic sensory organs) and physiological (increased life spans and development times, reduced metabolic rates and numbers of eggs) traits. These troglomorphic traits, exhibited on a global scale across diverse taxonomic groups, are one of the most powerful examples of habitat-driven convergence of form (Porter and Crandall, 2003) and one of the few demonstrated cases where convergent morphology can strongly mislead phylogenetic analyses (Wiens et al., 2003). The combination of regressive (lost) and progressive (enhanced) features found in cave-adapted faunas can lead to the existence of cryptic species, where two genetically different species are given one name based on morphological similarities. Even when species are diagnosed properly, convergent morphologies often lead to hypotheses of close evolutionary relationships among highly cave-adapted species, when in fact they represent

more distant lineages (Wiens et al., 2003). In some cases, troglomorphic morphologies have led to incorrect taxonomic designations above the species-level; molecular studies of the stygobiotic catfish *Prietella phreatophila* and *Prietella lundbergi* indicate that each is more closely related to species from different genera than they are to each other (*P. phreatophila* to *Ictalurus* species and *P. lundbergi* to *Ameiurus* species; Wilcox et al., 2004). In the absence of obvious morphological differences due to extreme convergence, molecular phylogenetic studies of troglobiotic and stygobiotic species have been successful at diagnosing the presence of taxonomic incongruencies based on cryptic morphologies, thereby changing our understanding of the distribution of subterranean fauna, and their relationships with each other and with epigean species (Chippindale et al., 2000; Parra-Olea, 2003; Buhay and Crandall, 2005).

Due to low population densities, the rarity of encountering some species, and the difficulties associated with collecting in some cave environments, our understanding of the distribution of cave fauna is also hampered by the difficulty in obtaining adult specimens, which are required for accurate species identification and taxonomic scrutiny. These constraints can be overcome by using molecular data to compare immature specimens to adult types of known species. For example, this approach has been used successfully with the *Cicurina* species from Texas, extending the range of the federally endangered *C. madla* to more than twice the number of previously reported caves; however, it is noted that this approach must remain a part of a balanced taxonomic approach by maintaining a taxonomic framework based upon multiple types of biological information (e.g., morphology, molecules, and ecology [Paquin and Hedin, 2004]).

DISPERSAL ABILITY

The phrase ''limited dispersal ability'' is common throughout the biospeleological literature (Holsinger, 1991; Coineau, 1994; Caccone and Sbordoni, 2001; Baratti et al., 2004). This assumption leads to hypotheses related to the isolation and speciation of cave faunas; limited dispersal abilities result in little to no genetic exchange between populations, allowing isolated populations to become genetically distinct, ultimately to the point of becoming different species. However, this dispersal assumption can be difficult to test empirically, particularly for species that may spend a significant amount of time traversing realms of the karst landscape and associated ground-water habitats that are inaccessible to the human researcher. For example, Buhay and Crandall (2005) used molecular studies of the mitochondrial 16S rRNA gene to investigate the stygobiotic *Orconectes* species in the Appalachians; larger than expected effective population sizes were used to infer the occurrence of a ground-water network unknown to humans but accessible to the crayfish. Furthermore, limited dispersal ability is a qualitative statement, providing no information useful for determining dispersal capabilities relative to habitat or other species. Yet, this tenet of limited dispersal is a central assumption to postulates of the importance of vicariance in subterranean distributions.

Using molecular methods, biospeleologists have begun to quantify the dispersal ability of subterranean fauna, in both relative and absolute terms. Comparing estimated gene flow among populations of cave and forest-dwelling cricket species, Caccone and Sbordoni (1987) demonstrate that cave species have lower rates of gene exchange than epigean species, with the degree of genetic differentiation in hypogean species correlated with the continuity of the limestone habitat. Similarly, in aquatic systems, population differentiation is related to habitat connectivity (Sbordoni et al., 2000). Given that ecological studies have shown that wide-ranging movements are possible for some stygobiotic species, particularly those capable of moving through interstitial habitats, such as ostracods (Danielopol et al., 1994), and that aquatic habitats have generally higher connectivity, stygobionts should have greater dispersal potential and capabilities than troglobionts (Lamoreaux, 2004).

At the heart of this issue are basic questions such as: What constitutes a cave population? What is the vagility of a particular species? How connected are these populations? Is habitat connectivity limiting dispersal? Was the habitat more or less connected in the past? These questions are affected both by intrinsic and extrinsic factors, making a complete answer dependent on understanding both the ecology (dispersal capability) and habitat (dispersal potential) of an organism. Molecular methods can address all of these questions, as the evolutionary history (including past and present dispersal events) is reflected in the genetic differences among populations, species, and genera of subterranean fauna. By delineating populations using genotypic clustering methods, the connectivity of a system can be investigated. For example, different caves in the same hydrologic system representing a single, randomly mating population can readily be identified. Conversely, patterns of genetic differentiation can be used to identify either unseen barriers to gene flow or gene flow across hypothesized geographic barriers; by estimating the phylogenetic structure and divergence times of the stygobiotic amphipod, *Niphargus virei*, Lefébure et al. (2006) found evidence for recent dispersal through apparent geographic barriers.

One of the few cases where molecular studies show strong support for an active migration (dispersal) model is in the anchialine gastropod, *Neritilia cavernicola* (Kano and Kase, 2004). *N. cavernicola* is a stygobiont found in anchialine caves on two islands in the Philippines situated 200 km apart. Genetic studies found no evidence of isolation between the islands, indicating the presence of a marine planktotrophic phase capable of migrating between the islands via ocean currents (Kano and Kase,

2004). Kano and Kase hypothesize that this active migration model, dependent on a larval stage tolerant of marine waters, may be common in anchialine stygobiotic fauna exhibiting disjunct insular distributions.

Migration rates and population structures may be the most interesting genetic parameters to estimate among troglobionts and stygobionts as a method to test the hypothesis that cave-adapted species are indeed poor dispersers relative to epigean organisms, and to quantify the differences in dispersal abilities among troglobionts and stygobionts, and among stygobionts from different subterranean habitats (epikarstic vs. phreatic).

VICARIANCE

In the classic model of vicariance, a once widely distributed ancestral species is fragmented within its range by an external (geological or climatic) event. This fragmentation leads to isolation of different segments (populations) of the species, allowing for genetic differentiation, and often speciation. Important to this model is timing; dating the event leading to fragmentation also provides the time since divergence of the derived set of species. Because this model is tied to external events, examples of vicariance-driven biogeography patterns are most obvious at large scales, including continental movements via tectonic events (Holsinger, 2005; Culver et al., 2007). One of the most widely used (and convincing) methods in biogeography to demonstrate these large scale vicariance patterns is to look for congruence in area cladograms constructed for different sets of species that have similar distributions. Basically, evolutionary relationships are reconstructed among diverse sets of species from a given area, and correlated with geography. If similar patterns of geographic patterning partitioned by evolutionary relationships emerge in many different taxa, there is strong evidence for large-scale vicariant events. Krejca (2005) proposed an even more rigorous test, where an *a priori* hypothesis of divergence patterns is created based on geologic history of a region, which is then tested by comparison to phylogenies constructed for the subterranean fauna of that region. Molecular phylogenetic methods assist these endeavors by making it possible to quickly generate cladograms for large numbers of populations and species. However, these types of broad studies using molecular data have not yet been widely employed to investigate subterranean biogeography (see Krejca, 2005 for an example).

The clearest examples of vicariant events in karst systems are 1) marine regressions (Culver et al., 2007) and 2) extirpation of surface populations from a species with both epigean and hypogean populations. However, in karst systems, patterns resulting from these types of vicariant events are virtually indistinguishable from a distribution resulting from dispersal (Culver et al., 2007). Therefore, perhaps the most promising way to investigate the relative influence of dispersal versus vicariance in karst

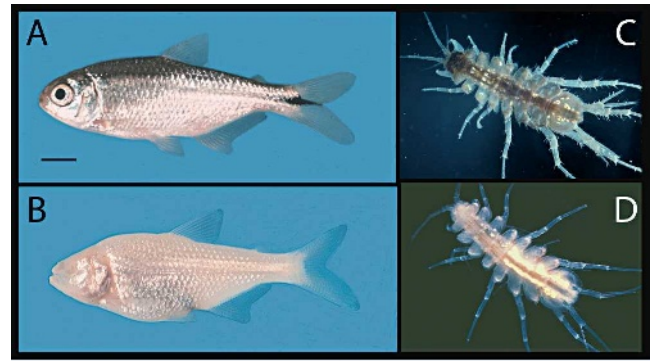

Figure 1. Epigean (A) and hypogean (B) forms of *Astyanax mexicanus*. Scale bar in A = 1 cm. Epigean (C) and hypogean (D) forms of *Asellus aquaticus* (photos provided by B. Sket). Specimen length in each panel = ca. 10 mm.

settings is to study species where both hypogean and epigean populations still co-exist or where closely related surface species have not yet been extirpated. However, even if a surface ancestor still exists, it can be difficult to identify due to the radical morphological changes present in the subterranean morphotype. Higher-level molecular phylogenetic studies offer increased resolution for comparisons across large geographic scales by providing more characters for phylogenetic analyses in organisms where convergence can make morphological characters difficult, and can help elucidate relationships among extant hypogean and epigean relationships (Cooper et al., 2002; Wiens et al., 2003).

Some of the best-studied examples of species with both epigean and hypogean populations include the isopod, *Asellus aquaticus*, and the fish, *Astyanax mexicanus* (Fig. 1). In these species, cave-adapted populations occur in the same drainages as epigean populations, offering the ability to investigate processes involved in the colonization and isolation of subsurface populations at the incipient stages of speciation.

Molecular studies of *A. aquaticus* incorporating estimates of population structure indicate that surface populations colonized caves to form stygobiotic populations three times within the Dinaric karst of Slovenia (Verovnik et al., 2004). Furthermore, estimates of divergence time indicate that the subsurface was invaded after the ancestral populations were isolated by vicariant fragmentation, demonstrating the genetic footprint ancestral surface population structures leave in hypogean populations and species. Similarly, molecular investigations of *A. mexicanus* indicate multiple origins of cave populations, representing at least two independent invasions from surface populations, with no measurable gene flow occurring between surface and cave populations (Dowling et al., 2002; Strecker et al., 2003). Again, the phylogenetic analyses indicate that the evolutionary history of the surface ancestors controls the genetic differentiation

of the hypogean populations, with three of the four cave populations investigated originating from an ancestral source different from the contemporary surface populations (Strecker et al., 2003, 2004).

Using molecular techniques, this pattern of multiple invasions into subterranean aquatic habitats has been documented for many stygobiotic species (Kano and Kase, 2004; Lefébure et al., 2006), including the stygobiotic dytiscid diving beetle fauna found in calcrete aquifers from western Australia (Cooper et al., 2002; Leys et al., 2003). The dytiscid fauna from this region has invaded the subsurface independently at least 26 times (18 times within the tribe Bidessini and eight times within the tribe Hydroporini; Leys et al., 2003) and based on divergence time estimation shows an evolutionary pattern consistent with a climatic vicariant event, where increasing aridity in the region extirpated a widespread epigean ancestor, driving the evolution of the subterranean diving beetles (climatic relict hypothesis; Cooper et al., 2002; Leys et al., 2003).

From these studies, the importance of the distribution and genetic structure of the ancestral surface species is emphasized in controlling subterranean biogeographic patterns and current genetic relationships. The difficulty lies in elucidating the influence of extinct epigean population structure on subterranean biogeography from processes occurring after the colonization of caves.

Divergence Times

Perhaps one of the most important parameters that can be estimated using molecular data is lineage ages. Placing dates on the origins of a particular cave-adapted lineage is an interesting and thought-provoking exercise, which leaves open the temptation to correlate divergence times with timing of cave colonization. However, it is necessary to remember that the age of a particular lineage does not necessarily correlate with the time of cave invasion (Verovnik et al., 2005). Particularly in highly fragmented surface habitats, epigean populations can be highly isolated, and therefore genetically divergent prior to cave invasion (see previous section [Verovnik et al., 2004]); this situation results in estimated lineage ages much older than time of cave occupancy, leading to misinterpretation of biogeographic determinants. Conversely, if dispersal and subsequent isolation are an important determinant of subterranean biogeography, it is possible for lineages to be younger than time of karst inhabitation. However, by knowing these stipulations and acting conservatively, estimating lineage ages is still a worthwhile endeavor. When combined with information on regional geologic histories, large-scale biogeographic patterns can be linked to either vicariant or dispersal events. Interestingly, most of the studies estimating divergence times using molecular clock methods have investigated stygobionts, and have postulated vicariance models or a mixed model of repeated range expansions and vicariant isolation (Table 1) (Ketmaier et al., 2003; Buhay and Crandall, 2005; Lefébure et al., 2006). In those studies where mixed models were invoked, however, vicariant events were related to larger scale phenomena while dispersal was linked to smaller scale phenomena within karst basins.

Many molecular studies of cave fauna have used gene sequence data to estimate divergence times based on molecular clocks, the assumption that DNA sequences change at a constant rate over time (Table 1) (Zuckerkandl and Pauling, 1965). With an estimate of sequence divergence between two species and a mutation rate in number of base pair substitutions per unit time, preferably calibrated to the taxon of interest, the age since the split can be inferred. There are a number of caveats associated with this type of analysis, however. When rates of evolution are compared within closely related species for the same DNA region, it is generally assumed they display clock-like behavior; however, most datasets appear to violate the clock model (Graur and Martin, 2004). Yet this assumption is rarely tested in studies of cave animals (see Caccone and Sbordoni, 2001 and Leys et al., 2003 for examples testing the assumption of a molecular clock) and the prevalence of many ancient cave adapted lineages may significantly violate any assumption of clock-like evolution. Second, usually mutation rates have not been estimated for the species of interest, so mutation rates from other, sometimes not so closely related, organisms are used. As this rate is used to convert sequence divergence to time, this is a critical assumption. Third, because mutation rates vary among genes, usually estimates are based on a single genetic marker. However, even considering all of these issues, in the absence of good fossil data or geologic events of a known age, molecular clock estimates provide a reasonable first approximation of time (Cooper et al., 2002). In a study of troglobiotic Bathysciine beetles from Sardinia using mitochondrial sequence data from the cytochrome oxidae I gene (COI), Caccone and Sbordoni (2001) illustrate how these caveats can be resolved. First, the assumption of a molecular clock was tested by investigating the linearity of evolution in the COI gene. Next, rates of COI evolution were empirically derived by calibrating sequence divergence to dates from well-defined geological events related to the splitting of the beetle lineages (Caccone and Sbordoni, 2001). These types of studies are extremely useful for calibrating rates of evolution in cave fauna, for investigating the evolution of the troglomorphic form, and for providing rate estimates for divergence time estimations in cave species where well-defined geological events correlating to lineage splitting are lacking.

More recent phylogenetic methods in estimating divergence times relax the assumption of clock-like sequence evolution and allow for multiple molecular markers to be incorporated into the estimate (Thorne et al., 1998; Sanderson, 2002; Thorne and Kishino, 2002; Yang, 2004), but these methods also require calibration points (i.e., fossils or geographic events associated with lineage splitting of known ages) to calculate divergence times

Table 1. Representative studies using molecular data to investigate the biogeography of troglobiotic and stygobiotic species.

Taxa	Genes[a]	Biogeographic model[b]	Region	Estimated Ages of Biogeographic Events	Reference
Troglobiotic					
Arachnida					
Araneae					
Nesticus	ND1	V, D, C	Appalachians, U.S.A.	…	Hedin, 1997
Hexapoda					
Coleoptera					
Ovobathyscioloa	COI	V	Sardinia, Italy	Sea level oscillations 16–5.5 Ma	Caccone and Sbordoni, 2001
Patriziella	COI	V	Sardinia, Italy	Pliocene climate change	Caccone and Sbordoni, 2001
Orthoptera					
Dolichopoda	16S, COI	V	Mediterranean	Pleistocene	Allegrucci et al., 2005
Crustacea					
Isopoda					
Littorophiloscia	COI	V, D	Hawaii, U.S.A.	…	Rivera et al., 2002
Hawaiioscia	COI	V, D	Hawaii, U.S.A.	…	Rivera et al., 2002
Stygobiotic					
Hexapoda					
Coleoptera					
Dytiscidae	COI, 16S, $tRNA_{leu}$, ND1	V	Western Australia	Late Miocene / Early Pliocene	Cooper et al., 2002; Leys et al., 2003
Crustacea					
Amphipoda					
Niphargus virei	COI, 28S	V, D	France	13 Ma	Lefébure et al., 2006
Isopoda					
Asellus aquaticus	COI, 28S	V, D	Dinaric Karst, Europe	8.9–2.9 Ma	Verovnik et al., 2004, 2005
Typhlocirolana	12S, 16S	V	Mediterranean basin	Tethyan events	Baratti et al., 2004
Stenasellus	COI	V, D	Corsica, Sardinia, Tuscany, Pyrenees, Italy	Miocene events and Quaternary glaciations	Kentmaier et al., 2003
Decapoda					
Orconectes	16S	V, D	Appalachians, U.S.A.	Cretaceous	Buhay and Crandall, 2005
Gastropoda					
Neritilia cavernicola	COI	D	Philippines	…	Kano and Kase, 2004
Vertebrata					
Teleostei					
Astyanax mexicanus	cytB, ND2	V, D	North and Central America	4.5–1.8 Ma	Dowling et al., 2002; Strecker et al., 2003; Strecker et al., 2004

[a] Gene region abbreviations: COI = cytochrome oxidase I, cytB = cytochrome B, ND1 = NADH dehydrogenase subunit 1, ND2 = NADH dehydrogenase subunit 2, 16S = 16S ribosomal RNA, 12S = 12S ribosomal RNA, $tRNA_{leu}$=leucine transfer RNA, 28S = 28S ribosomal RNA. All genes included in this table are mitochondrial, except for 28S which is a nuclear gene.

[b] V = vicariance, D = dispersal, C = competition.

across a phylogeny. These schemes are only recently being applied to subterranean biogeographic questions (Leys et al., 2003; Lefébure et al., 2006), and offer interesting research avenues that can correlate the age of a cave with phylogenetic estimates of hypogean divergence times. Using a method that relaxes the molecular clock assumption, Leys et al. (2003) investigated the timing of the transition from surface to subterranean life in the remarkable diversity of stygobiotic dytiscidae found in calcrete aquifers in Western Australia. By estimating divergence times between epigean and hypogean species, and between closely related species pairs that diverged after invasion of the subterranean calcrete habitat, a window was estimated for when the hypogean transition took place. The estimated ages for the eight pairs of species occurring in the same calcrete aquifers ranged from 3.6–8.7 Ma, representing the minimum age of the subterranean lineages. Estimates from hypogean species pairs occurring in different calcrete aquifers (representing independent subterranean invasions) provide a maximum age from 4.8–8.9 Ma, making the window of transition from surface to subterranean habitats from 8.9–3.6 Ma. Interestingly, there was a latitudinal pattern in divergence times coinciding with the onset of aridity, with species pairs from northern localities diverging earlier than southern localities (Leys et al., 2003).

The Future of Subterranean Biogeography

There is still much to learn about the processes driving current distributional patterns of organisms from caves and karst systems, and the combination of molecular techniques with the extensive work of subterranean biogeographers offers the potential to refine the questions being asked. Molecular phylogenetics and population genetics offer subterranean biogeography the ability to identify cryptic species, to link unidentifiable juvenile specimens to rare adult morphotypes to expand distributional ranges, to determine dispersal abilities via estimates of gene flow, population structure, and migration rates, and to estimate divergence times. Current molecular studies of hypogean populations overwhelmingly invoke either vicariant hypotheses, of either the ancestral surface or cave populations, or propose a mixed model, linking vicariance with range expansions (i.e., dispersal), to explain subterranean distributional patterns (Strecker et al., 2004; Verovnik et al., 2004; Buhay and Crandall, 2005; Lefébure et al., 2006); few studies have found evidence for a dispersal-only model of biogeography (Kano and Kase, 2004). However, at smaller scales (karst basins), molecular investigations of dispersal abilities offer insights into the connectivity of the subterranean realm. As molecular estimates of parameters such as population structure, migration rates, and divergence times, become more common, it will be possible to investigate how the disparities between troglobiotic and stygobiotic species affect genetic divergence and speciation, and to begin to quantify the dispersal abilities of cave organisms in general. The molecular biogeographical studies of subterranean fauna thus far have provided new perspectives into the distribution patterns of hypogean fauna, reinvigorating the vicariance versus dispersal debate. Finally, many of the molecular analyses used in biogeographic studies (population structure, gene flow, distributions) are also of supreme importance when considering conservation and management issues for subterranean fauna (Buhay and Crandall, 2005). Continued molecular investigations will provide information necessary for identifying the most imperiled cave species needing conservation.

Acknowledgements

Thanks to D.C. Culver, D. Fong, and H.H. Hobbs III for valuable insights and discussions regarding subterranean biogeography, and for the suggestion to write the manuscript in the first place. I am grateful to W.R. Jeffery and B. Sket for providing photographs and to D.C. Culver, K. Dittmar de la Cruz, A.S. Engel, and M. Pérez Losada for helpful comments regarding the manuscript.

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