Chapter 2
Phylogeny, Genes, and Hearing: Implications for the Evolution of Echolocation in Bats

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Keywords Bat hearing • Bat vision • Cetacea • Convergence • Laurasiatheria • Microchiroptera • Megachiroptera • Mammalian phylogeny • Molecular evolution • Olfaction • Sensory perception • Taste • Whales • Pseudogenes • Yangochiroptera • Yinpterochiroptera

2.1 Introduction

Of all the mammals, bats are arguably the most unusual, uniquely able to fly and also distinctively capable of laryngeal echolocation, enabling them to orient and move in complete darkness (Kunz and Fenton 2003). Being highly specialized mammals with unique adaptations, it is not surprising that the phylogenetic position and evolutionary history of the order Chiroptera has been argued and debated since they were first named by Blumenbach in 1779 (Simmons and Geisler 1998). Indeed, of all the mammalian orders, Chiroptera has been considered the most contentious in terms of its phylogenetic controversies. This has resulted in provocative conclusions about the evolution of echolocation and flight in mammals (see extensive reviews in Teeling et al. 2012; Jones et al. 2013).

To understand how echolocation has evolved, as well as the molecular basis of this spectacular trait, bat inter- and intra-ordinal relationships must first be resolved (see Figs. 2.1 and 2.2 for consensus relationships from molecular phylogenies). This has proven difficult over the past century because of conflicting phylogenies,

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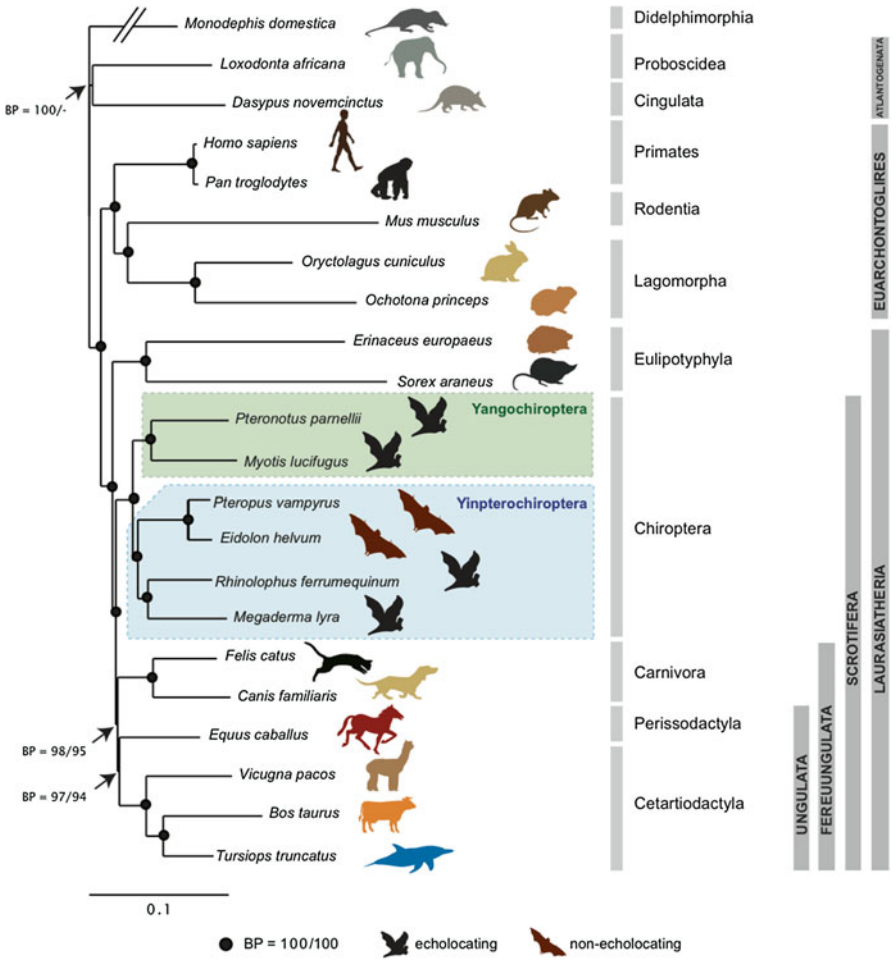

Fig. 2.1 The evolutionary relationships of Chiroptera among other mammalian lineages based on a large phylogenomic study with basal bat representatives (Taken from Tsagkogeorga et al. 2013 with permission)

arguably due to different data types, limited taxonomic sampling, and a poor fossil record (Teeling et al. 2012). However, the dawn of the "genomic and genetic era," in which molecular data have been used to build evolutionary trees, has seen great advances and paradigm shifts in our understanding of the evolutionary history of bats and other mammals (Figs. 2.1 and 2.2) (Springer et al. 2004; Jones and Teeling 2006). Within the past decade, we have approached a consensus stemming from analyses of large molecular and genomic data sets (Figure 2.1) (Meredith et al. 2011; Tsagkogeorga et al. 2013), and we have started to uncover the molecular basis of echolocation (Liu et al. 2010a, b; Parker et al. 2013).

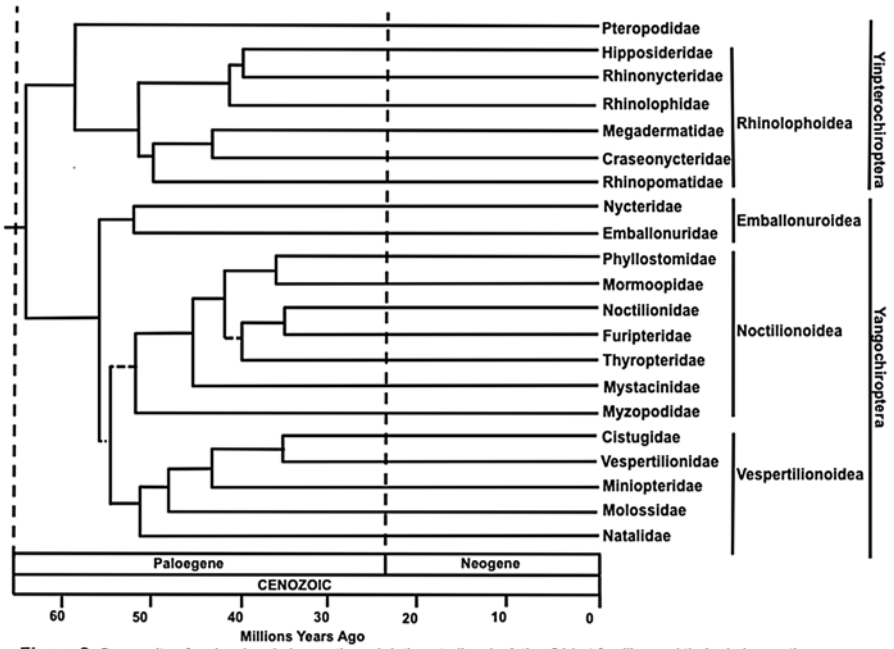

Fig. 2.2 A composite figure summarizing the consensus divergence times and family relationships among bats (Based on: Teeling 2009b; Lack et al. 2010; Meredith et al. 2011; Teeling et al. 2012 and references therein; Foley et al. 2014)

Currently within Chiroptera, there is agreement regarding the majority of interfamilial relationships based on large nuclear data sets (Teeling et al. 2005; Miller-Butterworth et al. 2007), mitochondrial, and nuclear whole genome studies (Meganthan et al. 2012; Tsagkogeorga et al. 2013). However, the position of the Pteropodidae, or Old World flying foxes, has recently been questioned by a large phenomic (morphological) data set (O'Leary et al. 2013), which contrasted with all published molecular-based phylogenies (Springer et al. 2013) and, therefore, questioned all current molecular-based conclusions regarding the evolution of laryngeal echolocation in bats.

Below, the consensus and conflict regarding bat evolutionary relationships is explored based on key phylogenetic studies over the past 15 years. The consequential conclusions regarding the evolution of bats' unique sensory perception are detailed. Using these consensus phylogenies as an evolutionary framework, the search for the genomic signatures of echolocation is described, and the spectacular unprecedented sequence convergence observed between echolocating bats and whales is highlighted. What is currently known about the molecular basis of echolocation is detailed, and the future steps required to link this trait with its genomic bases are explored. It is often argued that the evolution of one specialization will have consequences for other traits, and, in the case of echolocation, a "trade-off"

between the senses must occur (Jones et al. 2013). Here, the potential molecular sensory "trade-offs" between echolocation, vision, olfaction, and taste are explored. Finally, the next steps required to ultimately reach a universal consensus regarding bat phylogenetic relationships are discussed together with future directions for elucidating the genomic basis of echolocation in mammals and the extent of molecular trade-offs that occur in these remarkable species.

2.1.1 The Molecular Phylogenetic Position of Chiroptera Within Eutheria

Three key studies published in *Nature* and *Science* in 2001 provided the first robust support for four superordinal clades of mammals (Afrotheria, Laurasiatheria, Euarchontoglires, and Xenarthra; Table 2.1) and represented the largest genic and taxonomic data sets at that time (Madsen et al. 2001; Murphy et al. 2001a, b), ultimately changing the landscape of mammalian biology (Springer et al. 2004). The order Chiroptera was placed in the superordinal group Laurasiatheria along with carnivores (e.g., cats, dogs, seals), pangolins, cetartiodactyls (e.g., whales, cows), perrisodactyls (e.g., horses, rhinos), and eulipotyphylan insectivores (e.g., hedgehogs, shrews). This disassociated Chiroptera from its traditional morphology-based position in the superordinal clade Archonta, along with primates, tree shrews, and as the sister group to the flying lemurs, Dermoptera (Springer et al. 2004). Since these seminal papers, further molecular-based support for the four superordinal groups of placental mammals (Table 2.1), including Laurasiatheria, has come from many large phylogenomic data sets (e.g., Song et al. 2012; McCormack et al. 2012). Yet despite much congruence among studies, questions still remain regarding the position of the root of placental mammals (Morgan et al. 2013; Romiguier et al. 2013) and the branching patterns within Laurasiatheria (Meredith et al. 2011; Tsagkogeorga et al. 2013).

One of the largest studies in terms of taxonomic representation included 164 mammals representing up to 99 % of all recognized mammalian families for ~35,000 aligned nucleotide positions from 26 gene fragments (Meredith et al. 2011). Phylogenetic analyses and divergence time estimates provided high support for the four superordinal clades of mammals and estimated that Laurasiatheria originated approximately 85 million years ago (MYA) and that crown-group bats (i.e., all modern bats and their close fossil relatives) started to diverge approximately 66 MYA (Meredith et al. 2011). However, despite this comprehensive data set, it was still not possible to fully resolve the branching pattern within Laurasiatheria, arguably because of incomplete lineage sorting resulting from the rapid radiation and divergence of the laurasiatherian lineages (Springer et al. 2003; Romiguier et al. 2013). This has made it difficult to conclude which laurasiatherian family is the sister group to the bats and, therefore, has direct implications for interpreting how and when flight and echolocation originated in mammals.

Table 2.1 Details the composition of the four major placental mammal groupings

Superorder Laurasiatheria	Superorder Euarchontoglires
Order Chiroptera—e.g., bats	Order Rodentia—e.g., mice, rats
Order Perssiodactlya—e.g., horses, rhinos	Order Lagomorpha—e.g., rabbits, hares
Order Eulipotyphla—e.g., hedgehogs, shrews	Order Primates—e.g., Man, monkeys
Order Cetartiodactyla—e.g., whales, deer	Order Dermoptera—e.g., flying lemurs
Order Carnivora—e.g., dogs, lions, seals	Order Scandentia—e.g., tree-shrews
Order Philodota—e.g., pangolins	
Superorder Afrotheria	Order Xenarthra—e.g., armadillo, sloth
Order Afrosoricida—e.g., golden mole, tenrec	
Order Macroscelidea—e.g., elephant shrews	
Order Tubulidentata—e.g., aardvark	
Order Proboscidea—e.g., elephant	
Order Hyracoidea—e.g., hyrax	
Order Sirenia—e.g., manatee	

Analyses of shared retroposon (i.e., mobile DNA elements that originate from RNA molecules) insertion sites (Nishihara et al. 2006) and conserved non-coding elements (McCormack et al. 2012) found support for a sister group relationship between bats and horses, termed Pegasoferae (Nishihara et al. 2006). A recent taxonomically limited, whole genome phylogenetic study that included 10 mammals and 2 bat species (Zhang et al. 2013) also found support for a sister group relationship between bats and horses. However, this finding was contradicted by a more recent phylogenomic study that examined over 2,000 genes in 22 placental mammals including 6 bat species (Tsagkogeorga et al. 2013), the largest investigation of its kind to date. Using coalescent-based methods to accommodate the potential effects of incomplete lineage sorting, Tsagkogeorga et al. (2013) provided strong statistical support for Fereuungulata in which Carnivora is sister to an "ungulate" grouping containing Cetartiodactyla and Perrisodactyla, and bats are sister taxa to this group, a finding similar to other seminal phylogenetic/genomic studies (Figure 2.1) (Murphy et al. 2001b; Zhou et al. 2012).

2.1.2 Molecular Phylogenetic Relationships Within Chiroptera

Within the order Chiroptera itself, the past 15 years have seen considerable change and phylogenetic/systematic rearrangement resulting from large molecular studies (for a review see Teeling et al. 2012; for new family descriptions for Cistugidae see Lack et al. 2010; for Rhinonycteridae see Foley et al. 2014). Currently, there are over 1,260 species of bats (Simmons 2005) placed in 21 families. Figure 2.2 depicts these families, their interfamilial relationships, consensus divergence times, and also highlights nodes of controversy that differ between studies. Four superfamilial groups of echolocating bats are typically supported: Rhinolophoidea (Rhinolophidae,

Hipposideridae, Rhinonycteridae, Craseonycteridae, Megadermatidae, Rhinopomatidae); Vespertilionoidea (Vespertilionidae, Molossidae, Miniopteridae, Cistugidae, Natalidae); Emballonuroidea (Nycteridae, Emballonuridae); and Noctilionoidea (Myzopodidae, Mystacinidae, Furipteridae, Thyropteridae, Noctilionidae, Mormoopidae, Phyllostomidae). All non-laryngeal echolocating bats are placed in the family Pteropodidae.

Some discrepancies remain about the phylogenetic relationships between these superfamilies and, at times, their composition (Figure 2.2). Meredith et al. (2011) placed Emballonuroidea and Noctilionoidea as sister taxa, whereas Teeling et al. (2012) placed Emballonuroidea basal within the supordinal group Yangochiroptera, albeit with lower statistical support. The position of the monotypic Myzopoda can also differ between studies: Teeling et al. (2005) found support for a basal position for Myzopoda within the Noctilionoidea; however, Eick et al. (2005) and Meredith et al. (2011) supported a basal position for Myzopoda within the superfamily Vespertilionoidea. Within the superfamily Noctilionoidea, the phylogenetic position of Thyropteridae is still debated (Teeling et al. 2012; Jones et al. 2013), and potentially the Taphozinae should be elevated to familial level status as a sister family to Emballonuridae (Ruedi et al. 2012).

2.1.3 Yinpterochiroptera and Yangochiroptera

The most significant phylogenetic rearrangement in bats, especially in relation to the evolution of echolocation, pertains to the position of the non-echolocating family Pteropodidae. Molecular data consistently support a sister group relationship between the non-echolocating Pteropodidae and the echolocating superfamily Rhinolophoidea in the subordinal group Yinpterochiroptera (Teeling et al. 2000; Meredith et al. 2011). This is in stark contrast to previous morphologically based phylogenies, whereby all bats capable of laryngeal echolocation were placed into the monophyletic suborder Microchiroptera, and all non-laryngeal echolocating bats (Pteropodidae) were placed in the suborder Megachiroptera (Simmons and Geisler 1998; Teeling 2009a, b). This non-echolocating phenotype was considered basal within bats and laryngeal echolocation was considered to have evolved once (Teeling 2009a; Jones et al. 2013). In contrast, large molecular and mitochondrial data sets (see Teeling et al. 2012 for a review of previous studies; Meganthan et al. 2012) consistently supported a basal division in bats: Yinpterochiroptera (Pteropodidae + Rhinolophoidea) and Yangochiroptera (all other echolocating lineages) (Figure 2.2).

O'Leary et al. (2013) examined the phylogenetic relationships and divergence times among representative living and fossil mammals using a large supermatrix that combined 4,500 phenomic characters with DNA sequences for segments of 27 nuclear genes [based on the Meredith et al. (2011) molecular data set]. Using this large morphologically based data set and unusual/limited taxonomic sampling in bats (Fossil bats: *Onychonycteris*, *Icaronycteris*; Extant bats: *Pteropus*, *Rhinopoma*,

Pteronotus, *Myotis*, *Nycteris*, *Saccopteryx*), they recovered the classical Microchiroptera/Megachiroptera split (Simmons and Geisler 1998).

Within bats, morphological data have always supported a monophyletic Microchiroptera; however, this result is unsurprising given the known convergence of morphological characters in bats (Teeling et al. 2000, 2005). Bats are particularly prone to morphological convergences as they occupy a narrow ecological space and have constrained bauplans imposed by flight and echolocation. In the case of higher level relationships among placental mammals, there is a long history of discordance between morphological and molecular data (Springer et al. 2008), arguably resulting from rapid morphological mutations and high levels of morphological convergence, causing long-branch attraction and, therefore, inaccurate phylogenetic relationships (Springer et al. 2004, 2013). This problem can potentially be corrected for extant taxa by combining phenomic and genomic data sets, but genomic data are not available for most fossils and relatively few extant taxa have had their genomes sequenced (Springer et al. 2008). For these reasons it is likely that the results obtained by O'Leary et al. (2013) were particularly prone to the problem of long-branch attraction, which contributed to the erroneous support for Microchiroptera (Springer et al. 2013).

Indeed, Tsagkogeorga et al. (2013) is the only study to date to include genome data from echolocating species from both of the proposed suborders Yinpterochiroptera and Yangochiroptera. This study found unequivocal support for these groupings, and thus rejected the traditional subordinal bat clades of Microchiroptera and Megachiroptera (Figure 2.1). It follows that molecular-based phylogenies do not find support for the monophyly for Microchiroptera nor, by inference, a single acquisition of laryngeal echolocation in the ancestor of the echolocating lineages. Rather, molecular phylogenies suggest that laryngeal echolocation must either have evolved once in the ancestor of bats with subsequent loss in the "derived" pteropodids (Figure 2.3a) or have multiple acquisitions in the echolocating lineages (Figure 2.3b) within the two subordinal groups (Figure 2.3) (Jones et al. 2013). Despite the large molecular data sets (e.g., Parker et al. 2013; Tsagkogeorga et al. 2013) and the recovery of key transition bat fossils, such as *Onychonycteris finneyi* (Simmons et al. 2008; Veselka et al. 2010), this question still remains to be answered and represents a grand challenge in biology (see Teeling et al. 2012 for a review).

Potentially, more bat genomes sequenced and analyzed appropriately could uncover loss-of-function mutations in genes required for echolocation in pteropodid bats (Teeling et al. 2012), suggesting that they once had the ability to echolocate but lost it. Conversely, finding multiple genetic bases for echolocation in convergent echolocating bat lineages, but not in the pteropodids, would suggest independent, convergent gain of echolocation. However, before being able to take either of these approaches, the molecular bases for echolocation must be discovered. This is a difficult task, likened to finding the "Holy Grail" in this field (Teeling 2009a, b). However, recent whole genome comparative studies and different targeted gene approaches, focused on mammals with known auditory specializations for echolocation (bats and toothed whales), have finally started to uncover the molecular bases of echolocation.

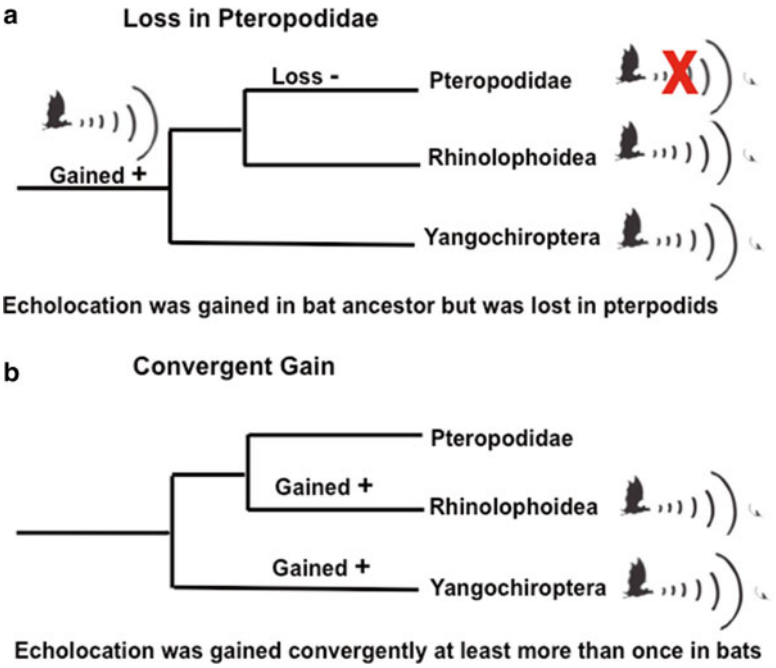

Fig. 2.3 Two alternative scenarios [(**a**) or (**b**)] regarding the gain/loss of echolocation in the pteropodid lineage

2.2 Auditory Specializations for Echolocation

Echolocation can be described as the production of sonar signals followed by comparison of the returning echoes for orientation, obstacle avoidance, and prey detection. It follows that echolocation necessitates a complex interplay of the vocalization, auditory, and neural systems, all of which are likely to have been subject to selection in the evolution of this sensory modality. Given the complexity of echolocation, it is especially remarkable that it has evolved not only in bats—possibly more than once—but also independently in the toothed cetaceans (order Odontoceti), making it a classic text book example of phenotypic convergence.

Attempts to identify the genes that function in echolocation in bats and cetaceans can be informed by studies of their respective key morphological, anatomical, and physiological specializations. Echolocating bats and cetaceans generate, emit, and receive sounds in contrasting ways. In bats, sound is generated in the larynx and, depending on the species, emitted through the mouth or nostrils. Although data on the laryngeal morphology of echolocating bats do not point to any gross adaptations, several features may represent specializations for generating ultrasonic pulses, such as the high degree of ossification of the larynx, calcification of the cricothyroid muscle, and extended vocal folds (Metzner and Schuller 2010; Carter 2014). In contrast,

sound generation in odontocetes seems to be initiated in the larynx but is subsequently propagated by specialized nasal plugs and a complex of tissues collectively known as the monkey lips and dorsal bursae (Cranford and Amundin 2003).

To date, most research into the adaptations of echolocating bats and cetaceans has focused on the auditory system (Vater and Kossl 2004; Surlykke et al. 2014). All echolocating mammals possess a broadly standard mammalian inner ear and middle ear. However, while the outer ears of bats consist of large mobile pinnae adapted for receiving and localizing incoming sound, cetaceans have lost their outer ears altogether and, instead, appear to receive and channel sound via fatty tissues in their jaws (Ketten 1997, 2000). The inner ear comprises the cochlea and semicircular canals, constituting the main auditory and vestibular structures, respectively. The cochleae and vestibular systems of echolocating bats are remarkably varied in structure and may compete for space within the petrous bone (Davies et al. 2013a). The relative length of the basilar membrane in the cochlea, and its number of turns, are seen to correlate with echolocation call parameters (Davies et al. 2013b). Comparative morphometric analyses show that horseshoe bats have among the longest relative basilar membranes, probably relating to the fine auditory tuning of their auditory foveae.

Compared to other mammals, the basilar membranes of echolocating bats are thickened and narrow at the basal turn of the cochlea, and the outer hair cells (OHCs) are short and tightly anchored to reinforced, supporting Deiter cells (Dannhof and Bruns 1991; Vater and Kossl 1996, 2004). All of these features occur in odontocetes (Ketten 2000; Vater and Kossl 2004). Similarly, the stereocilia of the hair cells are also unusually short in echolocating bats, possibly improving their sensitivity to incoming sound waves, although no comparative data are available from cetaceans (Vater et al. 1992; Yao et al. 2007). Overall, many of the structural modifications in cochleae of echolocating bats contribute to increased stiffness of the cochlear partition at the basal end, maximizing the transfer of energy from the OHCs to the basilar membrane (Russell 2014). Further apparent specializations for processing echolocation signals are seen in the ascending auditory pathway. For example, compared with other mammals, the brain stems of bats show a greater degree of hypertrophy in several structures, including the superior olivary complex (Grothe and Park 2000), anteroventral cochlear nucleus, and inferior colliculus (Pollak 1992; Covey and Caseday 1995). Parallel expansions of auditory nuclei and auditory regions have also been documented in the dolphin brain (Ridgway 2000).

2.2.1 The Molecular Basis of Hearing

Despite considerable knowledge of the phenotypic correlates of echolocation, almost nothing is known about its molecular basis. Yet, because echolocation is so closely linked to audition, the molecular machinery of echolocation is expected to largely overlap with that of hearing.

To date, much of our understanding of the molecular basis of hearing has come from two main approaches: mapping and animal models. In mapping studies, loci are

typically identified on the basis that they are linked to informative genetic markers. For example, much of our knowledge of hearing genes has come from identifying microsatellite loci or single-nucleotide polymorphisms (SNPs) that are associated with hearing impairment and then looking for genes near to these markers. By applying these so-called genome-wide association studies (GWAS) to sequence data from humans (Van Laer et al. 2010) and other species (Kluth and Distl 2013), the loci underpinning a large range of deafness conditions have been mapped to chromosomal positions, and, in many cases, the genes themselves have been identified. Once candidate genes have been identified, further insights into their role and importance in hearing have come from work on animal models, in particular mouse models (Leibovici et al. 2008).

As a consequence of these and related techniques, there have been remarkable advances in our understanding of the molecular basis of hearing and hearing loss. Indeed, over 100 genetic syndromes involving hearing loss (SHL) have been described, associated with mutations in one or multiple genes (Steel and Kros 2001; Petit 2006). Similarly, there are now over 100 recognized forms of "nonsyndromic hearing loss" (NSHL) in which hearing impairment is the only symptom present (Shalit and Avraham 2007). NSHL is also characterized by both allelic and locus heterogeneity. By convention, the names of loci associated with nonsyndromic hearing loss are prefixed by DFNX for X-linked deafness, DFNA for autosomal dominant deafness, or DFNB for autosomal recessive deafness. More details of the diversity of hearing genes discovered are available from recent reviews on the subject (Eisen and Ryugo 2007; Dror and Avraham 2010).

2.2.2 Studying the Molecular Basis of Hearing and Echolocation in Bats

Unfortunately, the conventional approaches for gene discovery outlined above are largely unsuitable for studying hearing and echolocation genes. In particular, mapping studies rely on the presence of intra-specific phenotypic variation, whereas bat and cetacean conspecifics do not tend to differ markedly in their echolocation call parameters. More fundamentally, mutations in hearing genes in bats and cetaceans are likely to result in lethal phenotypes. Therefore it is unsurprising that there are no reported cases of deafness in bats, although hearing loss has been reported in several stranded echolocating cetaceans (Finneran et al. 2007; Mann et al. 2010; Li et al. 2013). At the present time, conventional gene knockdown and knockout bat and cetacean models are limited, in part due to their slow rates of reproduction and the difficulties of culturing in the laboratory.

2.2.2.1 Candidate Gene Approaches

To date, nearly all attempts to identify genes involved in echolocation have taken a comparative approach, typically examining the molecular evolution of candidate genes that have been identified from humans, mice, and other organisms

(Jones et al. 2013). The rationale of such studies is that if genes show evidence of having undergone positive selection in echolocating taxa, then the gene products are likely to be of particular functional importance (e.g., Kirwan et al. 2013). While finding genes with roles in high-frequency hearing and echolocation might seem like looking for a needle in a haystack, candidate gene approaches have proven surprisingly successful in bats.

The gene encoding the forkhead transcription factor FOXP2 was one of the first putative echolocation genes to be studied. In humans, mapping studies show that mutations in the *FoxP2* gene are associated with deficits in aspects of speech and language, including orofacial coordination (Fisher et al. 1998; Lai et al. 2001), while molecular evolutionary analysis has revealed adaptive amino acid substitutions since the split with chimpanzees (Enard et al. 2002). From work on other non-echolocating species, *FoxP2* has been implicated in vocal learning in songbirds (Haesler et al. 2007) and in both ultrasonic vocalizations (Fujita et al. 2008, 2009) and auditory-motor learning in mice (Kurt et al. 2012). Given that echolocation in bats involves very rapid orofacial (or nasofacial) auditory-motor control, Li et al. (2007) tested whether *FoxP2* has been subject to molecular adaptation in the evolution of echolocation in bats. Gene sequence alignments revealed greater amino acid variation coupled with accelerated and divergent selection pressure in bats compared to other mammals, consistent with a role in echolocation. Further support for the involvement of *FoxP2* in echolocation comes from brain expression data, which show expression in the anterior cingulate cortex (ACC) and suprageniculate nucleus of echolocating bats at much higher levels than in Old World fruit bats (Metzner and Schuller 2010). Building on this result, Chen et al. (2013) have successfully applied lentivirus-based RNA interference (RNAi) to reduce the expression of *FoxP2* in the ACC, paving the way for behavioral studies in the future.

Additional insights into the molecular basis of vocalizations in bats come from experiments on echolocation pulse acoustics. Tressler et al. (2011) used a neurotoxin to target dopamine-producing cells in the basal ganglia and found that high striatal dopamine levels were associated with reductions in echolocation pulse amplitude, duration, and bandwidth. These results support a role for dopamine in the vocal control of echolocation, in line with findings from humans and rats that show an impact of striatal dopamine on the tone of the laryngeal musculature (Feng et al. 2009; Zarzur et al. 2010).

The majority of the studies of putative echolocation molecules to date have focused on hearing genes, in particular those implicated in a reduced sensitivity to high frequencies. Thus far, the best studied of these in bats has been the hearing gene *Prestin* (also known as *SLC26A5*; locus DFNB61); mutations in *Prestin* have been linked to autosomal recessive nonsyndromic hearing loss in humans (Liu et al. 2003). *Prestin* encodes a transmembrane solute carrier protein of the same name that is expressed and distributed in the OHCs (Zheng et al. 2000). In response to changes in membrane potential, *Prestin* undergoes voltage-dependent conformational changes that lead to electromotility of the OHCs; as such, *Prestin* is considered a key component of the cochlear amplifier that underpins the high sensitivity of the mammalian hearing apparatus (Liberman et al. 2002).

Li et al. (2008) undertook phylogenetic reconstructions of bats based on *Prestin* amino acid sequences and found that echolocating yinpterochropteran bats formed a well-supported clade with yangochiropterans to the exclusion of their true non-echolocating sister taxa, the pteropodid Old World fruit bats. The authors attributed this unexpected result to strong sequence convergence that was particularly evident in the cytoplasmic and extracellular domains of the protein, including the C-terminus. Follow-up studies that included cetacean *Prestin* sequences found even more dramatic convergence, this time between odontocetes and echolocating bats, together with evidence of molecular adaptation in multiple ancestral branches of echolocating taxa (Liu et al. 2010a, b). Building on these results, Liu et al. (2014) conducted functional assays and showed that that two parallel amino acid substitutions in the Prestin protein accounted for changes in the voltage-dependent membrane capacitance of cells, which in turn correlated with the frequency of best hearing sensitivity.

A strikingly similar signature of convergence in echolocating bats has been documented in the *Kcnq4* gene (Liu et al. 2011, 2012). In humans *KCNQ4* maps to locus DFNA2A, encodes a voltage-gated potassium channel protein, and is associated with nonsyndromic autosomal dominant deafness (Kubisch et al. 1999; Kharkovets et al. 2000). Liu et al. (2012) found eight parallel amino acid substitutions between the two groups of echolocating bats, while a similar study with slightly fewer taxa found four of these sites (Liu et al. 2011). As with Prestin, most of the parallel changes discovered were distributed in the cytoplasmic C-terminus of the protein. Immunofluorescence data from the mouse indicate that Kcnq4 expression follows both a longitudinal gradient, from the base to apex of the cochlea, as well as a radial gradient, from the IHCs to OHCs, with additional expression in the spiral ganglion neurons (SGNs) and vestibular hair cells (Beisel et al. 2005). The finding that the highest OHCs expression of Kcnq4 occurs at the apex, whereas the highest IHC and SGN expression occurs at the base, casts doubt on earlier speculation that Kcnq4 -associated deafness arises because of disrupted K^+ circulation in the OHCs; instead, this form of deafness might relate to problems of the IHCs and SGNs (Beisel et al. 2005).

Phylogenetic reconstructions based on the amino acid sequences of other hearing genes have also been found to recover erroneous well-supported groupings of laryngeal echolocating species. For example, Davies et al. (2012) reported similar findings from the genes *Tmc1* (locus DFNB7/11) and *Pjvk* (locus DFNB59) and Shen et al. (2012) from *Otof* (locus DFNB9) as well as *Cdh23* (locus DFNB12) and *Pcdh15* (locus DFNB23). The first of these, *Tmc1*, is expressed in both the IHCs and OHCs (Kurima et al. 2002) and encodes a transmembrane protein that functions in hair cell transduction and permeation (Kawashima et al. 2011; Pan et al. 2013). Davies et al. (2012) found particularly strong amino acid convergence at *Tmc1* between two bat species that have independently evolved high duty cycle echolocation with Doppler shift compensation: the horseshoe bat *Rhinolophus ferrumequinum* and the mustached bat *Pteronotus parnellii*. In contrast, comparisons of *Prestin* sequences from the same two taxa have revealed no such parallel changes (Shen et al. 2011).

The genes *Cdh23* and *Pcdh15* (encoding cadhedrin 23 and protocadhedrin 15, respectively) (Siemens et al. 2004; Ahmed et al. 2006) are both distributed in the tip links of the stereocilia and are thought to contribute to hair bundle motility. Shen et al. (2012) found parallel evolution in bats and dolphins, as well as positive selection in several key ancestral branches. The gene *Otof* encodes the protein Otoferlin, which has been shown to act as a calcium sensor mediating neurotransmitter release in cochlear hair cells, although its interactions with other proteins suggest additional functions (Zak et al. 2011). Real-time PCR has shown that in the echolocating common bent-winged bat (*Miniopterus schreibersii*) Otof expression levels are much higher (70-fold) in the auditory cortex compared with the cerebellum, whereas no such pattern was seen in the Old World fruit bat Leschenault's rousette (*Rousettus leschenaultii*) that does not possess laryngeal echolocation. Finally, data from mice suggest that the protein product of *Pjvk* (Pejvakin) probably has a role in the afferent auditory pathway (Delmaghani et al. 2006) rather than in the hair cells, which appear to be unaffected in mutant forms.

2.2.2.2 Genomics Approaches

Despite the success of some recent studies, candidate gene approaches undoubtedly often require considerable luck. New high throughput sequencing technologies offer the means to scale-up comparative approaches to genome scales, allowing thousands of loci to be studied at the same time (Brownstein et al. 2012; Yan et al. 2013). Zhang et al. (2013) tested for molecular adaptation in over 2,400 genes in David's myotis bat (*Myotis davidii*) and found significant positive selection in seven putative echolocation-related genes, including *Prestin*, *FoxP2*, and *Tmc1*, together with *Wnt8a*, *Fos*, *Mmp14* and *Dzip1*. Applying the same approach to the congeneric little brown bat (*Myotis brandtii*), Seim et al. (2013) analyzed 2,600 genes and found positive selection in two additional putative hearing genes, *Rgs7bp* and *Slc45a2*, as well as shared amino acid substitutions with the bottlenose dolphin (*Tursiops truncatus*) in two more hearing genes: *Trpv5*, mutants of which suffer from hair cell death, and *Nox3*, which is expressed in the inner ear and involved in the perception of gravity. Zhou et al. (2013) also investigated gene evolution in echolocating lineages, comparing 74 orthologs of putative hearing or vocalization genes in the Yangtze river dolphin (*Lipotes vexillifer*) and *M. lucifugus*. Of these, accelerated evolution was found in seven (*Prestin*, *Tmc1*, *Dzip1*, *Mmp14*, *Pax2*, *Wnt8a* and *Sparc*), of which parallel evolution was seen in the first three, as well as in 14 other genes, including *Myo15a*, *Otof*, *Notch1* and *Bmp4*.

Building on the findings of candidate gene studies, Parker et al. (2013) developed a bioinformatics pipeline to compare locus-wise support for competing phylogenetic hypotheses at a genome scale. They then used this method to identify all loci along a genome alignment that supported an erroneous grouping of either all unrelated echolocating bats or echolocating bats and the dolphin. This study showed that the strength of support for convergence for the trait echolocation was significantly stronger in hearing genes than in other genes. Moreover, the work identified

numerous other genes supporting convergence, including several known hearing and/or deafness genes (e.g., *Slc4a11*, *Coch*, *Itm2b*, *Ercc3*, and *Opa1*). Perhaps more interestingly, the results revealed numerous genes that support "echolocation convergence" but which are poorly known with no known roles in sensory perception, suggesting more investigation is needed. Finally, in the first attempt to identify regulatory sequences underpinning echolocation, Davies et al. (2014) screened ~82,000 mammal-specific conserved non-coding elements (CNEs), and looked for changes in evolutionary rates in those CNEs underlying auditory system development. The authors found clear differences between echolocating and non-echolocating taxa in the substitution rates of four CNEs associated with inner ear development, implying possible roles of these regulatory loci in echolocation.

2.2.2.3 Future Approaches

Future studies of the molecular basis of echolocation now have a rich resource of published genome data sets for bats (Seim et al. 2013; Tsagkogeorga et al. 2013; Zhang et al. 2013), and cetaceans (Gui et al. 2013; Zhou et al. 2013; Yim et al. 2014) with more genomes to be published soon (Genome 10K Community of Scientists 2009). An additional application of high-throughput sequencing that can provide strong verification of a role in organs or structures associated with echolocation is through the collection of expression data. In general, sequencing RNA transcripts (RNA-Seq) offers a cost-effective means of obtaining coding gene sequences, especially for specific tissues, and has already been used in several studies of bats (Shaw et al. 2012; Francischetti et al. 2013; Phillips et al. 2014; Huang et al. 2016). On the other hand, obtaining sufficient yields of non-degraded RNA from the cochleae of bats is technically difficult because of the high degree of mineralization of the cochlea and the small amounts of starting material. Such problems might partially be addressed by the emerging field of single-cell transcriptomics. Obtaining transcriptome data from the ears of cetaceans is arguably even more challenging because RNA degrades rapidly postmortem, thus precluding the use of stranded animals. Currently there is one published study of cochlear transcriptomes of bats, which examined the echolocating Rickett's big-footed bat, *Myotis ricketti* and the Old World greater short-nosed fruit bat, *Cynopterus sphinx* (Dong et al. 2013). Comparisons of expression profiles revealed 987 genes were significantly upregulated in the echolocating species, including 18 known hearing genes. Of these, only *Tmc1* has been studied in bats.

Unfortunately, genome-scale approaches also have problems; for example, they typically require considerable computational resources and present nontrivial analytical challenges. In comparative studies of multiple and often divergent species, aligning and assembling large volumes of short-read sequence data inevitably introduces errors, which if not detected and accounted for will appear as signals of molecular evolution in downstream analyses. Therefore, given that genome resequencing of bats has limited value in light of the absence of sufficient intraspecific natural or pathological phenotypic (i.e., echolocation) variation, perhaps future efforts to discover echolocation genes should focus on cases of very closely related

species that have evolved divergent call frequencies or even different systems of echolocation altogether. Indeed members of the mustached bat (*Pteronotus parnellii*) species complex (Clare et al. 2013) are the only examples of the genus to use high duty cycle echolocation, while there are many cases of sister-taxa/clades having undergone dramatic shifts in call frequency (Kingston and Rossiter 2004; Puechmaille et al. 2011). Finally, it is important to recognize that most sequencing approaches, including SNP-based assays, rarely take account of genome architecture, structural rearrangements, or copy number variation (CNV), the latter of which may present the genetic substrate for evolutionary phenotypic innovations (Perry et al. 2008; Paudel et al. 2013).

As studies of molecular evolution continue to add to the growing number of putative echolocation genes, there is a mounting need for functional assays. Mouse models have already been used for studying the impacts of bat gene sequences on limb development (Cretekos et al. 2008) and present enormous potential for studies of hearing. Transient knock down of hearing genes by RNA interference is also feasible (Chen et al. 2013); however, these approaches require access to the tissue of interest, precluding their use for studying cochlea-specific gene function.

The availability of whole genomes and large molecular data sets has enabled a genomic exploration of the consequences of extreme ecological adaptation, i.e., the acquisition of echolocation. Visual inspection of an echolocating bat (e.g., *Rhinolophus*) compared to a non-echolocating bat (e.g., *Pteropus*) reveals obvious morphological differences (small eyes and large nose leaves versus large eyes and no nose leaves) resulting from the acquisition of echolocation. Extreme adaptation typically causes loss of function in another trait. Therefore, these trade-offs in sensory perception should be mirrored within the genome. By using bats as a model of phenotypic plasticity and exploring the genetic bases of their unique and divergent sensory traits, the link between phenotype and genotype can be further elucidated, addressing another grand challenge in biology.

2.3 Are Sensory Trade-Offs Associated with the Evolution of Echolocation?

An assumption that is frequently held in sensory biology is that brains are energetically expensive to maintain, and, therefore, selection acting on the relative allocation of tissue among different regions of the brain is severe. Trade-offs in resource allocation may occur among brain regions that are specialized for specific sensory tasks (Harvey and Krebs 1990). For example, it may not be possible to evolve both sophisticated echolocation and vision; hence, enlargement of brain regions associated with echolocation may occur in tandem with a reduction in the sizes of other brain regions associated with vision. The "mosaic evolution" patterns that emerge independently in functional brain units may be subject to intense selection if brain size is constrained (Cooper et al. 1993), as is likely in bats. Echolocating bat species may need to be small to react quickly to rapidly returning echoes (Barclay and Brigham 1991) or to produce echolocation pulses at high repetition rates

(Jones 1994) using superfast muscles (Elemans et al. 2011; Ratcliffe et al. 2013). Indeed, 70 % of all echolocating bat species are under 20 g and 30 % are under 10 g (Jones 1996). Consequently, their small brains will have limited capacity for neuron populations. Trends in the evolution of brain size appear to be related to foraging strategy. Fast-flying bats may have undergone reductions in brain size over evolutionary time, while the demands of orienting in complex environments may have selected for increased brain size in maneuverable flyers (Safi et al. 2005).

Investigations of sensory trade-offs in animals have largely focused on morphological and anatomical traits. For example, Mexican cavefish (*Astyanax mexicanus*) have lost their eyes because they are of no use in the dark caves they inhabit, though they possess relatively large numbers of taste buds and neuromasts (sensory cells associated with long-distance, tactile-like sensing) and large olfactory bulbs compared with surface-feeding conspecific forms (Gunter and Meyer 2013). Similar trade-offs may be expected in bats if echolocation is more effective than vision for orientation in darkness. Bats that use laryngeal echolocation have relatively enlarged brain regions associated with audition, such as the inferior colliculus and the auditory cortex, while Old World fruit bats (Pteropodidae) have relatively enlarged brain regions associated with vision and olfaction (Dechmann and Safi 2009). Morphological and anatomical traits have a genetic basis, however, and investigating whether sensory trade-offs exist via a molecular evolutionary perspective has great potential.

Loss of function in sensory adaptations can arise through relaxed selection that leads to pseudogenization. Pseudogenes possess DNA sequences similar to related genes that produce functional proteins but have become non-functional from disabling mutations such as premature stop codons or frameshifts. Pseudogenes are the genetic equivalent of vestigial morphological traits, such as the non-functional hind limb bones in cetacean skeletons, and the detection of pseudogenes can provide clues about traits that perhaps were functional in ancestral relatives and became non-functional only more recently. For example, the detection of pseudogenes associated with vision may imply that visual systems have regressed over evolutionary time. Moreover, if pseudogenization is more prevalent in taxa that have evolved sophisticated echolocation, then perhaps sensory trade-offs can be implied as a result of the intense neural demands necessary for complex acoustic imaging. Below we review whether genes associated with olfaction, vision, and taste are more likely to have become pseudogenized in echolocating taxa and discuss whether the detection of sensory trade-offs at the genetic level is feasible in bats.

2.3.1 Olfaction

Olfaction is important in the lives of bats and is used by many species for communication and by some species for finding food (Altringham and Fenton 2003). Tetrapods possess two distinct olfactory systems that operate via different anatomical and neurobiological pathways, but they can overlap in function (see review in Hayden and Teeling 2014). The “main olfactory system” (MOS) is used for the

detection of volatile substances and involves olfactory sensory neurons in the nose that transmit information to the main olfactory bulb in the brain, then onwards to the olfactory cortex and other brain regions (Kishida et al. 2007). The "accessory olfactory system" (AOS) detects fluid-based stimuli (including pheromones) by the vomeronasal organ (VNO) located at the base of the nasal cavity. Nerves from the VNO connect to the accessory olfactory bulb from which signals are transmitted to the amygdala and the bed nucleus of the stria terminalis before being transmitted to the hypothalamus (Bhatnagar and Meisami 1998).

In the MOS, olfactory receptors (ORs) are expressed in the cell membranes of sensory neurons in the upper nasal epithelium. The ORs are G protein-coupled receptors and provide information that is translated by the brain into receptor codes representing specific scents (Rinaldi 2007). The OR genes comprise the largest gene family in mammalian genomes (Lindblad-Toh et al. 2005), accounting for 3–6 % of all protein coding genes (Niimura 2012). Animal species that use olfaction extensively typically possess large numbers of functional OR genes, and species that are less dependent on olfaction show high rates of pseudogenization in OR genes. Evidence for sensory trade-offs in other mammals includes high rates of loss of function by pseudogenization of OR genes in primates that evolved trichromatic color vision (Gilad et al. 2004), in the platypus that uses mechanoreception and electrodetection for finding prey, and in echolocating cetaceans (Niimura and Nei 2007; Hayden et al. 2010; Niimura 2012).

Transition to an obligate aquatic environment has resulted in significant modifications and reductions to chemosensory structures within cetaceans, including olfactory bulbs, olfactory nerves, and the cribiform plate, which are lacking postnatally in odontocetes and significantly reduced in mysticetes, compared with terrestrial mammals (McGowen et al. 2014). Indeed, whales have some of the lowest numbers of functional OR genes among mammals (Niimura 2012). However, additional loss of function mutations also may have resulted from the acquisition of echolocation. Odontocetes that echolocate have an extremely high proportion of OR pseudogenes (74–100 %); whereas, Mysticeti, none of which echolocate, have a lower proportion of OR pseudogenes (29–58 %) (McGowen et al. 2008, 2014).

Echolocation in cetaceans appears to have led to reduced investment in olfaction; however, no such trade-off is seen in bats. In fact, there is little evidence that echolocating bats have high rates of pseudogenization of OR genes (10–36 %) compared with other mammals, and the level of pseudogenization is similar in taxa that use laryngeal echolocation compared with the non-echolocating pteropodids. The lesser horseshoe bat, *Rhinolophus hipposideros*, uses echolocation involving Doppler-shift compensation yet shows only 10 % pseudogenization of OR genes, the lowest value among the 11 bat species studied (Hayden et al. 2010). Expanding the taxonomic representation, Hayden et al. (2014) generated and examined 5,517 OR genes from 27 bat species and still found no evidence of a sensory trade-off between echolocation and olfaction. Echolocating bats were not found to possess an OR gene repertoire that is significantly different from that of non-echolocating bats, and the variability in levels of OR pseudogenes could not be attributed to echolocation capabilities. However, there appears to be a trade-off between OR gene families,

arguably driven by frugivory (Hayden et al. 2014). In both the Phyllostomidae and Yinpterochiroptera, an increase in the proportion of genes in families OR 1/3/7 and OR 2/13 coupled with a loss of genes in family OR 5/8/9 coincided with frugivory, regardless of echolocation capabilities. This suggests that chemosensory trade-offs are occurring between different OR gene families in bats and are driven by feeding ecology rather than sensory modalities. A second family of receptors in the olfactory epithelium encoded by trace amine-associated receptors (TAARS) deserves further study in bats, especially as these may be associated with the detection of pheromones (Liberles and Buck 2006; Hayden and Teeling 2014).

The loss of the AOS in primates and birds correlates with the acquisition of trichromatic color vision and tetrachromatic color vision, respectively, suggesting sensory trade-offs have taken place (Zhang and Webb 2003). Loss of function in the AOS is widespread in bats, with evidence from anatomical research (Bhatnagar and Meisami 1998) that is consistent with findings of pseudogenization of the *Trpc2* gene (transient receptor potential cation channel, subfamily C, member 2) required for vomeronasal signal transduction (Zhao et al. 2011). Functional VNOs are found only in phyllostomid bats and in some species in the genera *Miniopterus* and *Pteronotus* (Bhatnagar and Meisami 1998). Loss of function in *Trpc2* has occurred several times independently in a number of bat lineages and is not related to whether taxa have dichromatic or monochromatic color vision or whether they echolocate. However, from a wider perspective, perhaps the loss of the AOS in most bat species might be related to the specialized neural demands imposed by both vision and echolocation at night. Extant whales have also lost their VNO and, as expected, show few or no functional VNO-related genes (McGowen et al. 2014); however, these losses have not occurred due to the acquisition of echolocation since the degradation of the VNO is estimated to have occurred before the split of Odontoceti and Mysticeti (McGowen et al. 2014).

2.3.2 Taste

Genes involved with the reception of bitter, sweet, and umami tastes have been sequenced in bats and cetaceans. The extent of pseudogenization of bitter taste (*T2r*) genes in bats does not differ much from those in the human and rat genomes (Zhuo et al. 2009). Zhao et al. (2010a) sequenced a 720 bp portion of the exon of the *Tas1R* gene (associated with the detection of sweetness) from 42 bat species from a wide range of families and found that the gene was pseudogenized only in three species of vampire bats where the detection of sweetness may be redundant. The *Tas1R1* gene associated with the detection of umami (savory or meatlike) taste was pseudogenized, not amplifiable, or absent in 31 bat species studied (Zhao et al. 2012), although it appears intact in all other mammals studied except the giant panda (Zhao et al. 2010b). Hence, the need for detecting umami taste (assumed to be used in detection of amino acids in nutritious foods; Herness and Gilbertson 1999) may be redundant in bats, though pseudogenization is common to echolocating and non-echolocating

taxa. Vampire bats are some of the only mammals known so far to lack multiple tastes, and this could be the result of sensory trade-offs associated with a wide range of neural demands from their functional AOS and their use of echolocation and thermoreception. In cetaceans, the tongue is characterized by very few taste buds, and bioinformatics analyses of the bottlenose dolphin genome have revealed many pseudogenized taste receptor genes (Jiang et al. 2012; McGowen et al. 2014). Feng et al. (2014) have confirmed that the genes responsible for tasting sweet, sour, umami, and bitter have all lost their protein-coding function in all cetaceans, whereas the function of the gene responsible for sensing salty tastes has been retained. Consequently, no trade-off can be linked to the acquisition of echolocation.

2.3.3 Vision

Vision is important in the lives of bat species, especially in non-echolocating pteropodids. Even in echolocating bats, vision is more effective than echolocation over long distances, although at the cost of reduced acuity (Boonman et al. 2013). Bat retinae are dominated by rods that confer sensitive monochromatic vision in dim-light conditions. The DNA sequences of the rod opsin (rhodopsin) were intact in 15 bat species, suggesting that the evolutionary advantages of rhodopsin are fundamental to all bat species (Zhao et al. 2009a). Color vision in bats is more complex, with some bat species resembling many other mammal species by being potentially dichromats with intact cone opsin genes sensitive to short (*Sws1*) and medium-to-long wavelengths (*Mws*/*Lws*) (Zhao et al. 2009b). Sensitivity to short wavelengths allows some bats to see ultraviolet light (Winter et al. 2003), and this could fulfill a range of functions, including the detection of flowers in dimly lit conditions. However, *Sws1* has become a pseudogene in all rhinolophid and hipposiderid bat species studied, as well as in some pteropodids, especially those that roost in caves (Zhao et al. 2009b). The lack of sensitivity to short wavelengths is supported by immunohistochemical evidence showing that the primary visual cortex does not respond to UV light in a pteropodid that roosts in caves (*Rousettus leschenaultii*) or in *Hipposideros armiger* (Xuan et al. 2012).

Perhaps the strongest evidence for sensory trade-offs comes from studies investigating genes associated with vision and audition in rhinolophid and hipposiderid bats. These bats use high-duty echolocation that is arguably the most specialized form of biosonar used in nature. Echolocation calls are constant frequency (CF), allowing the detection of fluttering targets in clutter and even allowing the bats to classify different types of fluttering targets and make adaptive decisions in prey selection (Koselj et al. 2011). Rhinolophid and hipposiderid bats also use frequency-modulated tails at the ends of their calls for target ranging, and they adjust the frequency of the CF component from call to call to compensate for Doppler shifts in echoes resulting from their flight speed (Trappe and Schnitzler 1982).

Other genes associated with visual perception have also become pseudogenes in rhinolophid and hipposiderid bats. Gja10 is a gap junction protein expressed in

retinal horizontal cells in mammals and is important in horizontal cell coupling. Irbp (encoded by the *Rbp3* gene) is the interphotoreceptor retinoid-binding protein critical for normal functioning of the visual cycle in most mammals. *Gja10* was pseudogenized in all ten rhinolophid and hipposiderid bat species studied but was intact in 14 of 19 species using low duty cycle echolocation and in six pteropodid species (Shen et al. 2013). *Rb3p* has also become a pseudogene in all six rhinolophid and hipposiderid bats investigated and in *Pteronotus parnellii*, which also uses high duty cycle echolocation with Doppler-shift compensation. The gene was nevertheless intact in three pteropodid species and in six of eight bat species using low duty cycle echolocation (Shen et al. 2013).

Although the loss of traits that are no longer necessary is well-documented in animals at both the anatomical and genetic levels (e.g., Carroll 2006), whether such "regressive evolution" (Jeffery 2009) occurs as a coevolutionary event associated with concomitant enhancement of other sensory modalities is more difficult to demonstrate. Perhaps the brains of bats are not as energetically demanding as those of primates, and sensory trade-offs may not be important during evolution of their brains (Dechmann and Safi 2009). Given their specialized echolocation abilities, the pseudogenization of *Rbp3*, *Gja10*, and *Sws1* in rhinolophid and hipposiderid bats is strongly suggestive of a sensory trade-off arising from an increased investment in echolocation at the expense of vision. Indeed, these taxa also show accelerated evolution of the hearing gene *Prestin* (Li et al. 2008) that may enhance their sensitivity to high frequencies (Liu et al. 2010a, b). Identification of such trade-offs is problematic because most bats are reliant on a wide range of senses in their lives, and so trade-offs need to be considered among a wide range of sensory modalities rather than between echolocation and vision alone.

2.4 Summary

Genetic and genomic data sets have provided great insights into our understanding of the position of bats among mammals, as well as the main sub-ordinal groupings of bats. Today there is overwhelming agreement, based on molecular data, that echolocating bats are paraphyletic, indicating that echolocation has either been lost in the Pteropodidae or has evolved multiple times in bats. In contrast, phylogenetic signals obtained from some morphological data sets have persisted to support the monophyly of echolocating bats. This latter arrangement is likely to arise from strong phenotypic convergence for echolocation that pervades multiple morphological characters, which appears to have confounded bat taxonomists for hundreds of years. Bat phylogenetics represents a "hot-bed" of future research to untangle which characters are homoplastic and which are not, ultimately advancing our understanding of the constraints of morphological evolution.

Additional problems probably stem from the limitations of current analytical approaches and knowledge regarding morphological evolution. For example, typically morphological characters are considered to be independent from each other,

whereas they might well be shaped by the same sets of underlying genes. Furthermore, uniting both morphological and molecular characters in a phylogenetic framework can lead to conflicting signals and violations of parameters; however, these analytical problems must be overcome to gain phylogenetic information from all the available data. As genome sequencing becomes cheaper and more accessible and more lineages of bats are included, it is inevitable that outstanding questions in bat sub-ordinal systematics will be resolved over time. However, analyses of genomic data also are fraught with analytical difficulties, and thus studying bats will enable the advancement of these methods required for all modern fields of biology. Arguably the greatest advances in the field of echolocation genomics among mammals will be in the genetic dissection of phenotypes, which, for bats and cetaceans, will include greater elucidation of the molecular basis of echolocation via functional assays, expression studies, and more complete locus-wide and taxonomic-wide surveys of molecular evolution. This is an exciting time for the study of bat evolution and mammalian echolocation. Indeed, within the next decade the study of some of nature's most highly specialized mammalian species, the bats and the whales, will ultimately illuminate links between genotype and phenotype never uncovered before.

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