JOURNAL OF AVIAN BIOLOGY 33: 5–14, 2002

Rapid, independent evolution of flightlessness in four species of Pacific Island rails (Rallidae): an analysis based on mitochondrial sequence data

Beth Slikas, Storrs L. Olson and Robert C. Fleischer

Slikas, B., Olson, S. L. and Fleischer, R. C. 2002. Rapid, independent evolution of flightlessness in four species of Pacific Island rails (Rallidae): an analysis based on mitochondrial sequence data. – J. Avian Biol. 33: 5–14.

Flightless rails were once ubiquitous in the avifauna of Pacific oceanic islands. Most species have become extinct since human colonization of islands began about 2000 years ago. In this study, we use mitochondrial sequence data to estimate the phylogenetic relationships and ages of four species of flightless insular rails in the genus *Porzana*: *palmeri*, from Laysan Island in the Hawaiian archipelago; *sandwichensis*, from the island of Hawaii; *monasa*, from Kosrae Island in Micronesia; and *atra*, from Henderson Island in the Pitcairn group. Although all four species survived into historic times, all but *atra* are now extinct. The optimal trees show that *palmeri* is descended from *Porzana pusilla*, a volant crake distributed widely throughout the Old World. *Porzana sandwichensis*, *P. monasa*, and *P. atra* are each descended from the lineage leading to *P. tabuensis*, a volant rail widespread in northern and eastern Australia and on islands north to Micronesia and the Philippines and east through Polynesia. Loss of flight appears to have evolved rapidly in these insular rails, based on both sequence divergence values and data on the ages of the islands. In the case of the Laysan Rail (*palmeri*), divergences including loss of flight probably evolved in less than 125,000 years.

B. Slikas and R. C. Fleischer, Molecular Genetics Lab, Smithsonian Institution, 3001 Connecticut Ave. NW, Washington, DC 20008, USA. E-mail: slikasb@nzp.si.edu. S. L. Olson, Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA.

The avian family Rallidae – the rails, gallinules, and coots – includes about 150 extant or recently extinct species (Taylor 1998). Rails have one of the most extensive distributions of any family of vertebrates, occurring worldwide and occupying a diversity of habitats. The family is remarkable for its high proportion of insular endemics. About 25% of extant or recently extinct rail species are confined to islands, and most of these rails are flightless (Taylor 1998). Flightlessness makes these rails particularly prone to extinction, caused by habitat destruction or introduced predators or disease. As many as 2000 species of flightless rails may have become extinct since human exploitation of islands began about 2000 years ago (Steadman 1995).

Loss of flight undoubtedly evolved repeatedly in the Rallidae, because islands harboring flightless rails are separated by substantial distances, and flightless birds have limited dispersal capability. Determining the relationships of flightless rails based on morphological traits is fraught with problems. Osteological differences within the few genera that have colonized islands are slight, and flightlessness causes drastic, convergent changes in skeletal proportions, further complicating osteological comparisons (Olson 1973a). In this study, we employ mitochondrial sequence data to estimate relationships among flightless and volant rails in the genera *Porzana* and *Amaurornis*. Our goals are to identify the colonizing lineages that gave rise to each insular species and to estimate the time since colonization. The latter provides an estimate of the time required for the evolution of flightlessness.

The genus *Porzana* includes 14 historically-known species of small- to medium-sized, short-billed crakes, distributed worldwide, including four flightless species

endemic to widely separated islands in the Pacific (Taylor 1998): the extant species *P. atra* (Henderson I.) and three recently extinct species (*P. monasa*, Kosrae I.; *P. palmeri*, Laysan I.; *P. sandwichensis*, Hawaii I.). The genus *Amaurornis* includes nine species of medium to medium-large rails in Africa, Asia, and Australasia. The morphological traits used to distinguish species in the genera *Porzana* and *Amaurornis* tend to vary continuously across the two groups, and several species have been shuffled between *Porzana* and *Amaurornis* in different classifications (Olson 1973a). Our sequence-based phylogeny clarifies some relationships within this vexing group.

Two of the flightless rails included in this study, *monasa* and *sandwichensis*, went extinct in the nineteenth century. Two specimens of *P. monasa* were collected in 1827–28 on Kosrae Island, in the Caroline island group, and the species has not been recorded since that time (Greenway 1967). *P. sandwichensis* was recorded and collected from both the windward and leeward coasts of the island of Hawaii. The species was last sighted in 1884; seven specimens exist in collections (Olson 1999). Introduced predators (rats, cats, dogs) probably played a major role in the extinction of both species (Taylor 1998, Olson 1999). *P. palmeri* was endemic to the island of Laysan, in the northwestern chain of the Hawaiian archipelago. The species was extirpated on Laysan by the mid-1920s, due to habitat destruction by introduced rabbits, but birds had been introduced to two islands in the Midway Atoll in the early part of the century and survived there until 1943, when rats were introduced, and the Laysan Rail was exterminated within two years (Olson 1999). *P. atra* still exists on Henderson Island in high densities (Jones et al. 1995). Henderson is a 37 km^2 raised reef island in the Pitcairn group, with no permanent fresh water, and the island has been unoccupied by humans since at least 1600 (Brooke and Jones 1995).

Methods

Sampling

Our data matrix includes twelve of the fourteen species currently placed in the genus *Porzana*, seven of nine species in the genus *Amaurornis*, and ten additional species of rails (Taylor 1998) (Appendix 1). We obtained 435 to 906 base pairs (bp) of sequence for at least one individual of each species, except *Porzana monasa* (279 bp). For ten species, we obtained at least partial sequence from a second individual, to check accuracy. To assess variation within two widespread, volant rails (*Porzana tabuensis*, *Porzana pusilla*), we obtained 198 bp of sequence from six individuals of each species (Appendix 1). For ten individuals in the data matrix, DNA was extracted from frozen or buffer-preserved (DMSO/$NaCl_2$) tissue samples. For the remaining 32 individuals, DNA was extracted from samples taken from museum study skins, either feathers or small slivers (about 1 mm × 3 mm) from the toe pad (Mundy et al. 1997).

Laboratory protocols

Extraction of DNA followed one of two protocols, depending on the type of sample. A standard extraction protocol was followed for frozen or buffer-preserved tissue (Slikas et al. 2000). Samples obtained from museum study skins were extracted using protocols designed for degraded or "ancient" sources of DNA (Cooper et al. 1992, Cooper 1994, Slikas et al. 2000). These extractions were performed in a designated "ancient DNA" laboratory, in a separate building from the primary genetics laboratory. No PCR amplifications are performed in the building housing the "ancient DNA" laboratory, and no PCR product is used or stored there. Stringent precautions were followed while extracting DNA and preparing PCR reactions involving "ancient" samples to avoid contamination (Slikas et al. 2000).

We obtained sequences from three non-contiguous mitochondrial gene regions: (1) a 320-bp fragment, including 85 bp from the 3-prime end of cytochrome oxidase II (COII), 75 bp representing the entire tRNA-lysine, and 161 bp representing most of the ATPase 8 gene (bases 8930 to 9240 in the *Gallus gallus* mitochondrial genome, Desjardins and Morais 1990); (2) a 314-bp fragment from the 5-prime end of cytochrome *b* (bases 14991 to 15304 in *Gallus gallus*); and (3) a 271-bp fragment from the 3-prime end of cytochrome *b* (bases 15765 to 16035 in *Gallus gallus*). All bases except the tRNA-lysine are protein-coding. For DNA extracts obtained from blood or tissue, we used primers CO2GQL and A6MNH (Lovette et al. 1998) to amplify the region including COII, tRNA-lysine, and ATPase8. For samples obtained from museum study skins, we amplified a smaller fragment that yielded 198 bp of sequence, including 37 bp of tRNA-lysine and 161 bp of ATPase8. We could not amplify the larger fragment from the study-skin samples, presumably because the DNA in the latter was too degraded. To amplify the smaller fragment, we used primers L9051 and H9241 (Table 1), where "L" and "H" designate the light and heavy mitochondrial DNA strands, respectively, and the numbers refer to the position of the 3-prime end of the primers in the *Gallus gallus* mitochondrial sequence (Desjardins and Morais 1990). The cytochrome *b* fragments were amplified with two sets of primers (Table 1): (1) L14957 or L15044 paired with H15331, H15252, or H15212 (5-prime end) and (2) L15764 or L15886 paired with H16065 (3-prime end). Amplified products were sequenced using standard cycle sequencing and

visualized with an ABI 373 automated sequencer. Sequences were aligned using Sequencher 3.1.1 (Gene Codes Corp, Ann Arbor, MI).

Data analysis

The program PAUP* (Swofford 1998) was used to calculate genetic distances and to estimate phylogenetic trees. First, we performed a partition-homogeneity test (Farris et al. 1994, 1995) in PAUP* to test for conflict in phylogenetic signal among three partitions of the data matrix: (1) COII and t-lysine (bases 1–159), (2) ATPase8 (bases 160–320), and (3) cytochrome *b* (bases 321–905). These partitions are based on differences in substitution rate among these gene regions (pers. obs.). The partition-homogeneity test showed no significant conflict among partitions, so phylogenetic analyses were conducted on the complete data matrix. Maximum-parsimony (MP) analyses were performed with a step-matrix imposed weighting transversions (tv) over transitions (ts), in order to model the more rapid accumulation of transitions (Lewin 1997). Analyses were run with two different weightings (tv:ts): 5:1 and 10:1. The actual ratio of transitions to transversions is difficult to estimate accurately, due to stochastic noise at low sequence divergence and saturation of transitions at high sequence divergence. For the sequence data in this study, the transition-transversion ratio ranges from 0.78 to 14.4, including all possible species pairs and excluding comparisons in which the number of transversions is zero. Our choice of step matrix weights falls within this range, so the selected values seem reasonable.

Maximum-likelihood (ML) analyses also were performed. The model and parameter values applied in the ML searches were selected by the following protocol. First, we evaluated the likelihood of our data matrix for each of ten trees, randomly selected from among the set of most-parsimonious trees (5:1 step matrix), under six different evolutionary models. The models differed in the number of substitution categories (nst = 2 or 6) and the type of rate variation across sites (none; gamma-distributed; site-specific, with different rates for non-coding regions and each codon position in coding regions). For each model, the parameter values were estimated to maximize the likelihood function. The simplest model and parameter values yielding a significantly higher likelihood than any other model (determined by the likelihood ratio test, Swofford et al. 1996) were selected for the maximum-likelihood search. The selected model included six substitution types (Rmatrix = 1.841, 8.175, 0.678, 0.382, 8.983) and gamma-distributed rate variation across sites (alpha = 0.29).

Heuristic searches were performed, with a random addition sequence of taxa (10 replicates), TBR branch swapping, and the MULPARS option in effect. To assess the strength of support for nodes, we used nonparametric bootstrapping (Felsenstein 1985) with the same search options as above, but with a simple addition sequence of taxa. We ran 1000 bootstrap replicates for three different maximum parsimony searches (characters unordered, 5:1 (tv:ts) step matrix, and 10:1 step matrix). We ran 100 maximum-likelihood bootstrap replicates.

Using PAUP* (Swofford 1998), we compared base composition and substitution rates and pattern between two protein-coding genes: ATPase 8 (161 bp) and cytochrome *b* (585 bp). We omitted the COII gene from comparisons, because we have only 85 bp of sequence. To assess the significance of differences between ATPase8 and cytochrome *b* in base frequencies and in the proportion of variable sites at each codon position, we employed chi-squared tests, using the frequencies observed in cytochrome *b* to calculate "expected" values for the ATPase 8 gene. To compare levels of saturation in the two genes, we plotted uncorrected pairwise distances for each gene against maximum-likelihood (ML) distances based on the full sequence matrix (906 bp). The latter distances serve as an approximation for time since divergence. The ML distances were calculated using the same model that we used in the search for the ML tree (see above).

Table 1. List of primers used in PCR and sequencing. The letters "L" and "H" in primer names designate the light and heavy strands of the mitochondrial genome, respectively, and the numbers refer to the location of the 3-prime end of the primer in the *Gallus gallus* mitochondrial genome (Desjardins and Morais 1990). Sequences are listed from 5-prime to 3-prime ends. Primers L14957 and H16065 were modified from Kocher et al. 1989.

L9051	CACCAGCACTAGCCTTTTAAG
H9241	TGGTCGAAGAAGCTTAGGTTCA
L14957	AAAAGCTTCCATCCAACATCTCAGCAT-GATGAAA
L15044	TACTAGCCATGCACTATACTGCAGA
H15331	AAACTGCAGCCCCTCAGAATGATATTT
H15252	GTAGGATGATTCCTGTGTTTC
H15212	GAGCCGTAGTAGAATCCTCGGC
L15764	TCAATCCCAGAYAAACTAGGAGG
L15886	CTATTCTGAACCCTGGTCGCCAA
H16065	GGAGTCTTCAGTCTCTGGTTTACAAGAC

Results

Sequence characteristics

The data matrix includes 906 characters, but five contiguous non-coding sites in tRNA-lysine were excluded from all analyses, due to alignment uncertainty. Among the 901 included characters, 401 characters are variable, and 307 characters are parsimony informative. Among the parsimony informative characters, 63 are at first codon positions, 28 are at second codon positions, 204

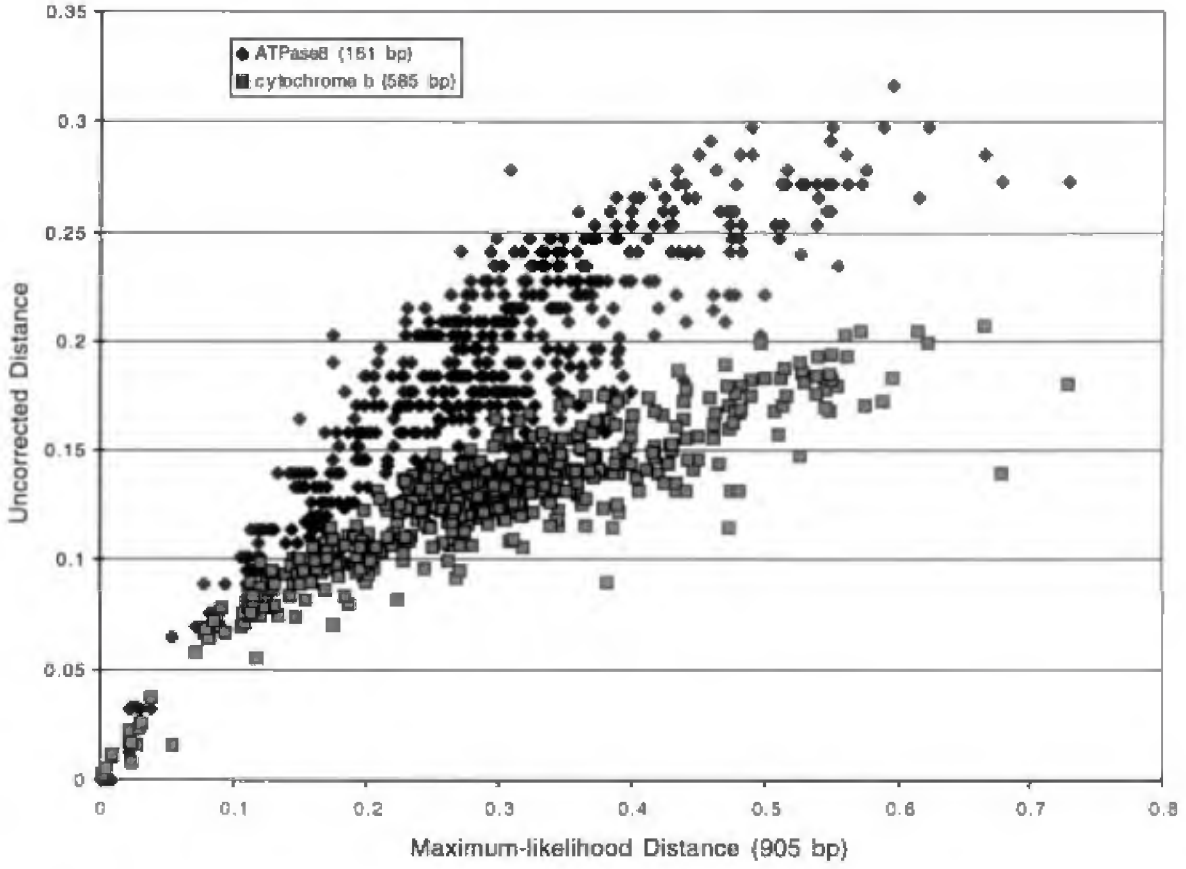

Fig. 1. Plot of uncorrected sequence divergence (y-axis) versus maximum-likelihood-corrected distances (x-axis) for all species pairs. The diamonds (◆) represent uncorrected distances based on ATPase8 gene sequence (161 bp), and the squares (■) represent uncorrected distances based on cytochrome *b* sequence (585 bp). Maximum-likelihood distances (x-axis) were calculated in PAUP* (Swofford 1998), using the full sequence data matrix (906 bp) and assuming the same model as in the search for the ML tree (Rmatrix = 1.841, 8.175, 0.678, 0.382, 8.983; alpha = 0.29).

characters are at third codon positions, and 12 are at non-coding positions.

The sequences exhibit a skewed base composition. Across all sites, nucleotide proportions are as follows: adenine, 30%; cytosine, 33%; guanine, 12%; thymine, 25%. Nucleotide proportions differ among codon positions, and third codon positions show the greatest skew in base composition, with a marked deficiency of guanine (3.5%) and thymine (12%). A chi-squared test showed no significant variation in base composition among taxa ($p = 0.999$), with all sites included. These patterns are typical for mitochondrial coding regions in birds (Slikas 1997, Sheldon et al. 1999, Espinosa de los Monteros 2000).

Sequence evolution

Our data matrix includes sequence from three different mitochondrial coding genes: COII, ATPase8 and cytochrome *b*. We have only a small amount of sequence from COII (85 bp), so we omitted this gene from the comparisons described below. The ATPase8 and cytochrome *b* genes differ in base composition and rates and pattern of nucleotide substitutions. The difference in base composition is significant (chi-squared test, $p < 0.025$), with the ATPase8 gene showing a greater deficiency in guanine. The ATPase8 gene accumulates mutations more rapidly than cytochrome *b*. Across the taxa in this data set, 56.5% of the 161 sites in the ATPase8 gene are variable, while only 43.3% of the 585 sites in cytochrome *b* are variable. Variable sites are more evenly distributed across codon positions in ATPase8, with 29.2% at first codon positions, 25.0% at second codon positions, and 45.8% at third codon positions, compared to 22.5%, 7.1%, and 70.4%, respectively, in cytochrome *b*. The difference between the genes in the proportion of variable sites at each codon position is significant (chi-squared, $p \ll 0.001$). The more even distribution of substitution rates across the ATPase8 gene results in less saturation (i.e., fewer instances of multiple substitutions at sites) than in

cytochrome *b*. When uncorrected distances based on each gene region are plotted against a distance corrected for multiple substitutions (Fig. 1), the distances based on cytochrome *b* clearly plateau earlier than distances based on ATPase8, suggesting more rapid saturation of the former. The ATPase8 gene has a higher overall substitution rate than cytochrome *b*, but substitutions are distributed more evenly across sites, resulting in less saturation.

Phylogeny

A maximum parsimony search with characters unordered yielded 420 most-parsimonious (MP) trees. Searches with either a 5:1 or 10:1 weighting of transversions over transitions (tv:ts) yielded 75 MP trees. The semistrict consensus trees from the latter two searches are identical, and majority-rule bootstrap consensus trees (1000 replicates) are also identical in topology and similar in bootstrap percentages. The tree from the maximum-likelihood search is congruent with the MP trees in all nodes with greater than 70% bootstrap support (Fig. 2).

Based on the optimal trees from both MP and ML searches, neither the genus *Porzana* nor *Amaurornis* is monophyletic as currently defined. The sequence data give strong support for three distinct clades including species in *Porzana* and *Amaurornis*: (1) *Amaurornis phoenicurus*, *A. isabellinus*, *A. olivaceus*; (2) *Porzana porzana*, *P. carolina*, *P. fluminea*; (3) all other species in *Porzana* and *Amaurornis* included in this data set. These three clades each have strong bootstrap support. Two species, *Aenigmatolimnas marginalis* and *Poliolimnas cinereus* consistently group with the trio of *A. phoenicurus*, *isabellinus*, and *olivaceus*, but bootstrap

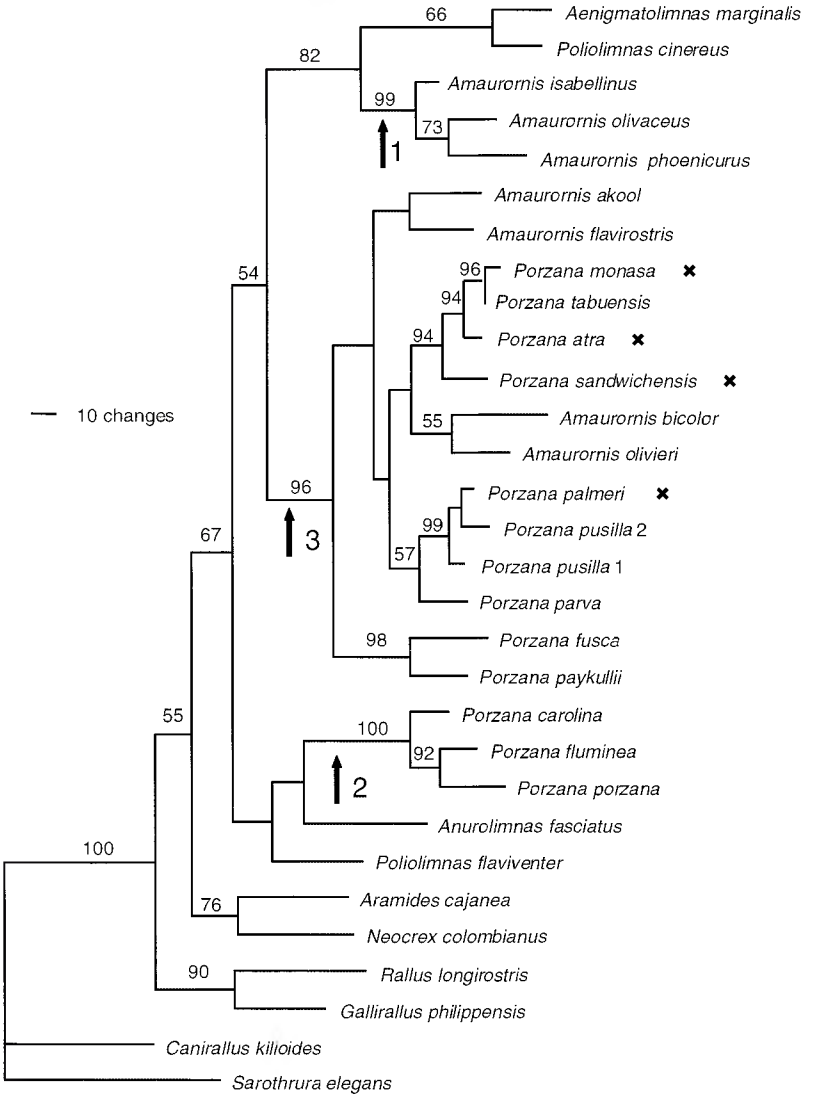

Fig. 2. Maximum likelihood tree resulting from a heuristic search using PAUP* (Swofford 1998), with 6 substitution types (Rmatrix = 1.841, 8.175, 0.678, 0.382, 8.983) and gamma-distributed rate variation across sites (alpha = 0.29). Numbers on the branches are bootstrap percentages based on 100 replicates. The symbol "✖" marks flightless species. Vertical arrows with numbers (1, 2, 3) designate clades discussed in the text.

Table 2. The upper half of each matrix shows uncorrected distances based on 198 bp of mitochondrial DNA sequence including portions of tRNA-lysine (37 bp) and ATPase 8 (161 bp) (bases 9052 through 9240 in the *Gallus gallus* mitochondrial genome, Desjardins and Morais 1990). Standard errors are listed in the lower half of each matrix (Nei 1987). All substitutions were transitions. Two individuals of *P. sandwichensis* yielded identical sequence.

	pusilla 1	*pusilla* 2	*pusilla* 3	*pusilla* 4	*pusilla* 5	*pusilla* 6	*palmeri* 1	*palmeri* 2	*palmeri* 3
pusilla 1	–	0.0310	0.0154	0.0308	0.0310	0.0205	0.0154	0.0205	0.0154
pusilla 2	0.0124	–	0.0465	0.0413	0.0191	0.0310	0.0362	0.0413	0.0362
pusilla 3	0.0088	0.0151	–	0.0359	0.0425	0.0256	0.0205	0.0256	0.0205
pusilla 4	0.0124	0.0142	0.0133	–	0.0364	0.0205	0.0154	0.0205	0.0154
pusilla 5	0.0124	0.0098	0.0144	0.0134	–	0.0255	0.0315	0.0378	0.0315
pusilla 6	0.0101	0.0124	0.0113	0.0101	0.0113	–	0.0154	0.0205	0.0154
palmeri 1	0.0088	0.0134	0.0101	0.0088	0.0125	0.0088	–	0.0051	0.0000
palmeri 2	0.0101	0.0142	0.0113	0.0101	0.0137	0.0124	0.0051	–	0.0051
palmeri 3	0.0088	0.0134	0.0101	0.0088	0.0125	0.0088	*	0.0051	–

	tab. 1	*tab.* 2	*tab.* 3	*tab.* 4	*tab.* 5	*tab.* 6	*atra*	*monasa*	*sand.*
tabuensis 1	–	0.0051	0.0051	0.0051	0.0051	0.0154	0.0308	0.0313	0.0615
tabuensis 2	0.0051	–	0.0000	0.0000	0.0000	0.0205	0.0256	0.0310	0.0564
tabuensis 3	0.0051	*	–	0.0000	0.0000	0.0205	0.0256	0.0310	0.0564
tabuensis 4	0.0051	*	*	–	0.0000	0.0205	0.0256	0.0310	0.0564
tabuensis 5	0.0051	*	*	*	–	0.0205	0.0256	0.0310	0.0564
tabuensis 6	0.0088	0.0101	0.0101	0.0101	0.0101	–	0.0462	0.0467	0.0769
atra	0.0124	0.0113	0.0113	0.0113	0.0113	0.0150	–	0.0691	0.0615
monasa	0.0125	0.0124	0.0124	0.0124	0.0124	0.0151	0.0181	–	0.0748
sandwichensis	0.0172	0.0165	0.0165	0.0165	0.0165	0.0190	0.0172	0.0188	–

support for this larger clade is weak, and the relationships among the *Amaurornis* trio, *Aenigmatolimnas*, and *Poliolimnas cinerea* differ between the maximum parsimony and maximum likelihood trees.

Within the clade including most species in *Porzana* and *Amaurornis* (Fig. 2, clade 3), some relationships have strong bootstrap support, including the pairing of *P. fusca* and *P. paykulli* and the pairing of *P. pusilla* and *P. palmeri*. A clade including *P. monasa*, *P. tabuensis*, *P. atra*, and *P. sandwichensis* also has strong bootstrap support. The latter clade is completely resolved, and the nodes have strong bootstrap support and are consistent across analyses.

Within clade 3, interspecific sequence divergence values range from 2.0% (*P. tabuensis* to *P. atra*) to 14.8% (*A. akool* to *P. monasa*), with a mean of 9.86% (SD = 2.78%). Within clade 1, divergences range from 6.13% to 8.77%, with a mean of 7.74% (SD = 1.41%), and within clade 2, divergences range from 8.00% to 10.3%, with a mean of 8.93% (SD = 1.21%). Among these three clades, divergence values range from 12.5% to 19.6%. Sequence divergence values between the two outgroups (*Sarothrura elegans*, *Canirallus kioloides)* and all other species in the data matrix range from 16.1% to 21.4% for *Canirallus* (mean = 18.1%, SD = 1.28%) and 18.4% to 23.2% for *Sarothrura* (mean = 20.1%, SD = 1.25%).

We calculated intraspecific divergence values for selected species based on 198 bp of sequence (37 bp of tRNA-lysine, 161 bp of ATPase8; Table 2). Among six individuals of *Porzana pusilla*, divergences averaged 3.01% (SD = 0.92%), and all substitutions were transitions. Three individuals of *P. palmeri* differed by a single transition in one individual, in a non-coding region. Between *pusilla* and *palmeri*, the average divergence over all pairwise comparisons was 2.42% (SD = 0.92%), smaller than the average divergence among individuals of *pusilla*. Five individuals of *Porzana tabuensis* differed by a single transition at a non-coding site. The sixth individual, from the Philippines, differed by an additional three transitions, giving an average divergence of 1.95% (SD = 0.22%) between this individual and other representatives of *tabuensis*. Two individuals of *Porzana sandwichensis* yielded identical sequence to one another.

Discussion

Origins of flightless rails

The flightless rails included in this data set occupy islands scattered throughout the Pacific, from the Hawaiian archipelago (Laysan, Hawaii), to eastern Polynesia (Henderson I.), to Micronesia (Kosrae I.). Given the isolation of these islands, loss of flight undoubtedly evolved independently in each insular endemic species. This hypothesis is consistent with the topology of our optimal trees (Fig. 2). If colonization had occurred between islands, then the island rails would pair as sister taxa. Instead, the flightless rails branch off from volant lineages. In our optimal trees, the flightless Laysan rail *Porzana palmeri* is sister to the volant Baillon's crake *Porzana pusilla*, and the flightless Kosrae rail *Porzana monasa*, Henderson rail *Porzana atra*, and Hawaiian rail *P. sandwichensis* are descended from the lineage leading to the volant *P. tabuensis*.

Both *P. pusilla* and *P. tabuensis* are plausible candidates for colonizing islands in the Pacific. *P. pusilla* is distributed widely across Europe and Asia and is migratory across most of its range (Cramp and Simmons 1980). The sister relationship between *pusilla* and *palmeri* was suggested previously by Olson (1973a, b), based on overall similarity in size and plumage pattern between the two species. *P. tabuensis* has a widespread but patchy distribution, extending from the Philippines, eastern Australia, New Guinea, and New Zealand across the Pacific to Micronesia and Polynesia. Interestingly, *tabuensis* does not occur on Kosrae Island (Micronesia) or Henderson Island (eastern Polynesia), islands that harbor(ed) its flightless relatives, but it does breed on Oeno Island, a neighboring island to Henderson in the Pitcairn group (Holyoak and Thibault 1984).

The flightless rails *P. monasa* (Kosrae I.) and *P. atra* (Henderson I.) are similar to the volant *P. tabuensis* in size, and all three species share a dark plumage, with no or very little mottling or barring (Ripley 1977). Olson (1973a) cited references suggesting that both *atra* and *monasa* are descended from *tabuensis*. Our optimal trees and sequence divergence values suggest that all three species are closely related, with *monasa* being the more recent descendant from the *tabuensis* lineage. The Hawaiian rail *P. sandwichensis* is more similar to the Ruddy-breasted crake *P. fusca* in overall plumage color and pattern. Olson (1973a) proposed that *sandwichensis* might be descended from *fusca*, but our optimal trees strongly support *tabuensis* as the ancestor of *sandwichensis*, implying that the similarity to *fusca* in plumage color is either convergent or symplesiomorphic. The heavily melanized plumage of *tabuensis*, *ater*, and *monasa* is atypical among rails (Taylor 1998), and the melanization might mask a lighter, patterned plumage, such as found in *sandwichensis*. Among the species in our data set, *sandwichensis* is the earliest offshoot from the *tabuensis* lineage.

All the flightless rail species included in this study formerly were placed in monotypic genera. Olson (1973a) argued that these genera were unwarranted, being based primarily on features associated with loss of flight. He suggested that the monotypic genera *Aphanolimnas* (*monasa*), *Nesophylax* (*ater*), *Porzanula* (*palmeri*), and *Pennula* (*sandwichensis*) be synonomized with *Porzana*. The optimal trees based on our mitochondrial sequence data support this recommendation (Fig. 2).

Age of flightless rails

The average sequence divergence (Table 2) within the volant and widespread *P. pusilla* (3.01%, n = 6) is greater than the average divergence between *pusilla* and its insular derivative, *palmeri* (2.42%). These divergences are based on 198 base pairs of sequence (37 bp of tRNA-lysine, 161 bp of ATPase8). In fact, in optimal trees including all six individuals of *P. pusilla* and three of *palmeri*, *pusilla* is paraphyletic with respect to *palmeri*, although bootstrap support for relationships within this group is weak. The relatively low sequence divergence between the volant *pusilla* and flightless *palmeri*, compared to levels of divergence within *pusilla*, suggests that the two species separated recently. The island of Laysan, located to the northwest of the main Hawaiian islands, is about 20 million years old, but it is now a low island, with a maximum elevation of only 12 m above sea level. During the last interglacial, about 125,000 years ago (Harmon et al. 1983), sea levels were 5 to 9 m higher than at present, probably closer to 9 m higher (Olson 1999). At that time, Laysan Island could not have been inhabited permanently by terrestrial organisms, because it would have been completely inundated in any serious storm. The colonization of Laysan Island by *P. pusilla*, the ancestor of the flightless endemic *palmeri*, probably occurred within the past 125,000 years. During this relatively brief time span, the Laysan rail evolved all of its distinctions from *P. pusilla*, including flightlessness.

In contrast to *P. pusilla*, *P. tabuensis* shows little within-species sequence divergence (Table 2), despite its broad distribution. Based on a sample size of six individuals from across its range, only the individual from the Philippines is distinctive, having greater than 1% divergence from other individuals. The low sequence divergence and the uniformity in body size and plumage color and pattern found across much of the range of *tabuensis* suggest that this species recently expanded from a small population, with a more restricted distribution. This hypothesis is supported by paleontological data. *P. tabuensis* is absent from the fossil record of islands in its current range in eastern Polynesia (Steadman 1987) and New Zealand (Millener 1981) until about 2000 years ago, after the arrival of Polynesians. The apparently recent spread of *P. tabuensis* across the Pacific might be causally related to the widespread, human-induced extinction of flightless rails on Pacific islands during the past 2000 years, suggesting that flightless endemic rails were able to exclude the volant *tabuensis* from colonizing islands in the past. This scenario contradicts a basic premise of the taxon-cycle hypothesis (Ricklefs and Cox 1972). The island-adapted flightless rails apparently were not at a competitive disadvantage with respect to newly arriving potential colonists.

The maximum age of each flightless taxon can be no greater than the length of time that its island has been above sea level, although a taxon's age might be much less, because organisms can colonize at any time after emergence of an island. *P. monasa* was endemic to Kosrae, a high, volcanic island about 4 MY old (Whittaker 1998). *P. atra* is endemic to Henderson Island, a raised coralline island (37.5 m above modern sea level)

that has been sub-aerial for about 380,000 years (Blake 1995). Both *monasa* and *atra* have about the same sequence divergence from *tabuensis* (Table 2: to *monasa*, 2.97%; to *atra*, 2.60%), suggesting that both taxa are similar in age. In fact, based on our optimal trees, *monasa* is more recently derived from *tabuensis* than is *atra*, despite its slightly higher sequence divergence. The age of Henderson Island gives an upper limit of 380,000 years for the age of the endemic *P. atra*; presumably *P. monasa* is similar in age. Alternatively, *monasa* could be a much older taxon (because Kosrae is an older island), with a slower rate of sequence evolution, but the latter explanation is less parsimonious.

P. sandwichensis was endemic to the island of Hawaii, a volcanic island about 430,000 years old (Carson and Clague 1995), similar in age to Henderson Island. However, the sequence divergence between *tabuensis* and *sandwichensis* (5.68%) is substantially greater than that between *tabuensis* and its other two flightless derivatives. Also, our optimal trees imply that *sandwichensis* split from the *tabuensis* lineage earlier than did *monasa* or *atra*. The sequence of *sandwichensis* differs from that of *tabuensis* by an average of 12 substitutions (range, 11–15; SE = 0.24). Using the lower bound of the 95% confidence interval, at least 11 substitutions differentiate *tabuensis* and *sandwichensis*. If these 11 substitutions accrued since the two species split, and if *sandwichensis* originated on Hawaii, then the implied substitution rate for this gene region is 13% per MY. This rate is exceptionally high, and either or both of the two assumptions used in calculating the rate might be wrong. First, it is possible that some of the divergence between the two species is due to ancestral polymorphism in *tabuensis*. Although our survey of genetic variation across the range of *tabuensis* (Table 2) showed very little intraspecific divergence, the sample size was small. In addition, current variation might not represent variation in the recent past, particularly if the species underwent a population bottleneck. A second possibility is that *tabuensis* colonized an older island in the Hawaiian chain, and *sandwichensis* is descended from another endemic taxon on this older island. No fossil remains of volant rails have been found in the Hawaiian islands, except the distinctive Hawaiian coot, but bones of several species of flightless rails, all referable to *Porzana*, have been collected from the islands of Hawaii, Maui, Molokai, Oahu, and Kauai (Olson and James 1991).

Very few data are available on the life-history characteristics of *monasa* (Taylor 1998) or *sandwichensis* (Olson 1999). Somewhat more information was recorded for *palmeri* (Olson 1999), and *atra* has been the subject of a thorough study (Jones et al. 1995). No consistent differences in plumage, breeding habits, feeding habits, voice, or behavior are apparent between the insular flightless rails and their continental relatives. Insular rails, both volant and flightless, tend to have smaller clutch sizes than rails living on continents. For example, populations of *P. tabuensis* on islands have smaller clutches, on average, than populations on the Australian continent (Taylor 1998). Jones et al. (1995) note a striking similarity in life-history traits, behavior, and voice between *atra* and its volant ancestor, *tabuensis*.

Evolution of flightlessness

The topology of our optimal tree (Fig. 2) implies that each of the four flightless rails included in this data set evolved flightlessness independently, because each flightless taxon branches off from a volant ancestral lineage, and no two flightless species pair as sister taxa. The evolution of flightlessness also occurred rapidly, in less than 500,000 years in at least three flightless species (*P. palmeri*, *monasa*, *atra*), based on the combined evidence of sequence divergence values between volant lineages and their flightless descendants and the length of time that islands harboring flightless rails have been above sea level. This pattern of repeated evolution of flightlessness in insular rails and the rapidity of the transition from volant to flightless suggests that this evolutionary change is driven by selection, as opposed to genetic drift (see also Trewick 1997). If the loss of flight is favored by selection, then it must be advantageous; in other words, maintenance of the ability to fly must exert a cost. So, to explain the evolution of loss of flight, it is necessary to invoke a cost, such as the physiological cost of maintaining flight muscles (Olson 1973b, McNab 1994, Roff 1994).

Interestingly, if the trait of flightlessness is mapped onto our optimal tree to trace its evolution (Fig. 1), the most parsimonious mapping requires a transition from flightless (*P. monasa*) to volant (*P. tabuensis*). If Dollo parsimony (Farris 1977) is applied, such that the transition from flightless to volant is disallowed, then the optimal mapping of the trait involves a transition from volant to flightless for each flightless island taxon, as expected. This result highlights the necessity of using accurate transition probabilities in mapping characters onto trees to track their evolution (Omland 1999).

Acknowledgements – We heartily thank the institutions listed in Appendix 1 and their curators and collection managers for providing tissues that made this study possible. In particular, we thank V. Loskot (ZISP) for providing feathers from the two existing specimens of *P. monasa* and G. Barrowclough (AMNH) and R. Symonds (UMZC) for allowing us to sample specimens of the extinct *P. sandwichensis*. We thank S. Birks (UWBM), A. Cibois (MNHN), D. Dittmann (LSUMZ), N. Rice (ANSP), and D. Willard (FMNH) for providing information about voucher specimens. This work was supported by the National Geographic Society, Friends of the National Zoo, and the Abbott Fund (Smithsonian Institution). B. Slikas was supported by a Smithsonian Molecular Evolution postdoctoral fellowship.

References

Blake, S. G. 1995. Late Quaternary history of Henderson Island, Pitcairn Group. – Biol. J. Linn. Soc. 56: 43–62.

Brooke, M., de, L. and Jones, P. J. 1995. The diet of the Henderson fruit dove *Ptilinopus insularis*. I. Field observations of fruit choice. – Biol. J. Linn. Soc. 56: 149–165.

Carson, H. L. and Clague, D. A. 1995. Geology and biogeography of the Hawaiian Islands. – In: Wagner, W. L. and Funk, V. A. (eds), Hawaiian biogeography. Smithsonian Inst. Press, Washington, DC, pp. 14–29.

Cooper, A. 1994. DNA from museum specimens. – In: Herrmann, B. and Herrmann, S. (eds), Ancient DNA. Springer, New York, pp. 149–165.

Cooper, A., Mourerchauvire, C., Chambers, G. K., Vonhaeseler, A., Wilson, A. C. and Pääbo, S. 1992. Independent origins of New Zealand moas and kiwis. – Proc. Natl. Acad. Sci. USA 89: 8741–8744.

Cramp, S. and Simmons, K. E. L. (eds). 1980. The birds of the western Palearctic. Vol. 2. – Oxford Univ. Press, Oxford.

Desjardins, P. and Morais, R. 1990. Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. – J. Mol. Biol. 212: 599–634.

Espinosa de los Monteros, A. 2000. Higher-level phylogeny of Trogoniformes. – Mol. Phylogenet. Evol. 14: 20–34.

Farris, J. S. 1977. Phylogenetic analysis under Dollo's Law. – Syst. Zool. 26: 77–88.

Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1994. Testing significance of congruence. – Cladistics 10: 315–320.

Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1995. Constructing a significance test for incongruence. – Syst. Biol. 44: 570–572.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. – Evolution 39: 783–791.

Greenway, J. C. 1967. Extinct and vanishing birds of the world. – Dover Publ., NY.

Harmon, R. S., Mitterer, R. M., Kriausakul, N., Land, L. S. and Schwarcz, H. P. 1983. U-series and amino-acid racemization geochronology of Bermuda: implications for eustatic sea-level fluctuation over the past 250,000 years. – Palaeogeogr. Palaeoclimatol. Palaeoecol. 44: 41–70.

Holyoak, D. T. and Thibault, J. C. 1984. Contribution à l'étude des oiseaux de Polynésie Orientale. – Memoirs du Muséum National D'Histoire Naturelle Série A. Zoologie 127.

Jones, P., Schubel, S., Jolly, J., Brooke, M., de, L. and Vickery, J. 1995. Behaviour, natural history, and annual cycle of the Henderson Island Rail *Porzana atra* (Aves: Rallidae). – Biol. J. Linn. Soc. 56: 167–183.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. – Proc. Natl. Acad. Sci. USA 86: 6196–6200.

Lewin, B. 1997. Genes VI. – Oxford Univ. Press, New York, NY.

Lovette, I. J., Bermingham, E., Seutin, G. and Ricklefs, R. E. 1998. Evolutionary differentiation in three endemic West Indian warblers. – Auk 115: 890–903.

McNab, B. K. 1994. Energy conservation and the evolution of flightlessness in birds. – Am. Nat. 144: 628–642.

Millener, P. R. 1981. The Quaternary avifauna of the North Island, New Zealand. – Ph.D. thesis, University of Auckland, NZ.

Mundy, N. I., Unitt, P. and Woodruff, D. S. 1997. Skin from feet of museum specimens as a non-destructive source of DNA for avian genotyping. – Auk 114: 126–129.

Nei, M. 1987. Molecular evolutionary genetics. – Columbia Univ. Press, New York, NY.

Olson, S. L. 1973a. A classification of the Rallidae. – Wilson Bull. 85: 381–446.

Olson, S. L. 1973b. Evolution of the rails of the South Atlantic islands (Aves: Rallidae). – Smithsonian Contrib. Zool. 152: 1–53.

Olson, S. L. 1999. Laysan Rail (*Porzana palmeri*) and Hawaiian Rail (*Porzana sandwichensis*). No. 426. – In: Poole, A. and Gill, F. (eds). The birds of North America. The Birds of North America, Inc., Philadelphia, PA.

Olson, S. L. and James, H. F. 1991. Descriptions of thirty-two new species of birds from the Hawaiian Islands. Part 1, non-Passeriformes. – Orn. Monogr. 45: 1–88.

Omland, K. E. 1999. The assumptions and challenges of ancestral state reconstructions. – Syst. Biol. 48: 604–611.

Ricklefs, R. E. and Cox, G. W. 1972. Taxon cycles in the West Indian avifauna. – Am. Nat. 106: 195–219.

Ripley, S. D. 1977. Rails of the world. – Godine, Boston.

Roff, D. A. 1994. The evolution of flightlessness: is history important? – Evol. Ecol. 8: 639–657.

Sheldon, F. H., Whittingham, L. A. and Winkler, D. W. 1999. A comparison of cytochrome *b* and DNA hybridization data bearing on the phylogeny of swallows (Aves: Hirundinidae). – Mol. Phylogenet. Evol. 11: 320–331.

Slikas, B. 1997. Phylogeny of the avian family Ciconiidae (storks) based on cytochrome *b* sequences and DNA–DNA hybridization distances. – Mol. Phylogenet. Evol. 8: 275–300.

Slikas, B., Jones, I. B., Derrickson, S. R. and Fleischer, R. C. 2000. Phylogenetic relationships of Micronesian white-eyes based on mitochondrial sequence data. – Auk 117: 355–365.

Steadman, D. W. 1987. Two new species of rails (Aves: Rallidae) from Mangaia, southern Cook Islands. – Pacific Sci. 40: 27–43.

Steadman, D. W. 1995. Prehistoric extinctions of Pacific island birds: Biodiversity meets zooarchaeology. – Science 267: 1123–1131.

Swofford, D. L., Olsen, G. J., Waddell, P. J. and Hillis, D. M. 1996. Phylognetic inference. – In: Hillis, D. M., Mable, B. K. and Moritz, C. (eds), Molecular systematics, 2nd edn. Sinauer, Sunderland, MA, pp. 407–514.

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. – Sinauer, Sunderland, MA.

Taylor, B. 1998. Rails: A guide to the rails, crakes, gallinules, and coots of the world. – Yale Univ. Press, New Haven, CT.

Trewick, S. A. 1997. Flightlessness and phylogeny amongst endemic rails (Aves: Rallidae) of the New Zealand region. – Phil. Trans. R. Soc. Lond. B 352: 429–446.

Whittaker, R. J. 1998. Island biogeography: ecology, evolution, and conservation. – Oxford Univ. Press, New York, NY.

(*Received 22 January 2001, revised 8 May 2001, accepted 15 May 2001.*)

Appendix 1. List of taxa and associated data. The museum catalog number refers to the voucher specimen associated with the tissue or blood sample, except for underlined numbers. The latter are tissue or blood catalog numbers. Catalog numbers marked with a dagger have no associated voucher. Abbreviations are as follows: AMNH, American Museum of Natural History; ANSP, Academy of Natural Sciences of Philadelphia; BMNH, British Museum of Natural History (Tring); BYUH, Brigham Young University, Hawaii; FMNH, Field Museum of Natural History; LSUMZ, Louisiana State University, Museum of Zoology; MNHN, Museum National D'Histoire Naturelle; ROM, Royal Ontario Museum; UMZC, University Museum of Zoology Cambridge; USNM, United States National Museum (Smithsonian); UWBM, University of Washington Burke Museum; ZISP, Zoological Institute St. Petersburg; ZMUC, Zoological Museum, University of Copenhagen.

Species	Collection locality	Museum catalog number	Source of DNA	# base pairs sequenced		
				COII+t-lys	ATP8	cyt *b*
Sarothrura elegans	Burundi, Africa	FMNH 346189	tissue	159	161	585
Canirallus kioloides	Madagascar	FMNH 345622	tissue	159	161	585
Anurolimnas fasciatus	Ecuador	ANSP 186054	tissue	159	161	585
Gallirallus philippensis	Philippines	ZMUC P556†	blood	159	161	462
Rallus longirostris	Louisiana, USA	LSUMZ 134706	tissue	159	161	585
Aramides cajanea	Brazil	USNM B06856	tissue	159	161	585
Amaurornis akool	China	USNM 303008	study skin	37	161	312
Amaurornis isabellinus	Celebes	USNM 248144	study skin	37	161	200
Amaurornis olivaceus	Solomon Islands	UWBM AWK 1443	tissue	159	161	507
Amaurornis phoenicurus	Philippines	FMNH 358237	tissue	159	161	585
Porzana porzana	Egypt	USNM 552588	study skin	37	161	312
Porzana fluminea	NSW, Australia	AMNH 546062	study skin	37	161	312
Porzana carolina	Louisiana, USA	LSUMZ 130486	tissue	159	161	585
Amaurornis flavirostris	Botswana	USNM 527276	study skin	37	161	384
Amaurornis olivieri	Madagascar	BMNH 1931.8.18.1790	study skin	37	161	462
Amaurornis bicolor	China	USNM 296113	study skin	37	161	435
Porzana parva	Azores	MNHN CG1987, # 741	study skin	37	161	348
Porzana pusilla 1	Egypt	USNM 550863	study skin	37	161	498
Porzana pusilla 2	NSW, Australia	USNM 278020	study skin	37	161	406
Porzana pusilla 3	Madagascar	BMNH 89.11.3.347	study skin	37	161	0
Porzana pusilla 4	New Caledonia	BMNH 97.10.30.561	study skin	37	161	0
Porzana pusilla 5	New Guinea	BMNH 1953.17.49	study skin	37	161	0
Porzana pusilla 6	India	MNHN CG 1970, # 910	study skin	37	161	0
Porzana palmeri 1	Laysan Island	UMZC 15/Ral/45/a/1	study skin	37	161	462
Porzana palmeri 2	Laysan Island	BMNH 1898.4.29.29	study skin	37	161	0
Porzana palmeri 3	Laysan Island	ROM 37402	study skin	37	161	0
Porzana sandwichensis	Hawaii Island	AMNH 546232	study skin	37	161	348
Porzana sandwichensis	Hawaii Island	UMZC 15/Ral/38/2/1	study skin	37	161	348
Porzana fusca	Philippines	FMNH 344923	tissue	159	161	585
Porzana paykulli	Thailand	USNM 306620	study skin	37	161	435
Porzana tabuensis 1	NSW, Australia	USNM 153063	study skin	37	161	384
Porzana tabuensis 2	Chatham Islands	UMZC 15/Ral/44/m/6	study skin	37	161	348
Porzana tabuensis 3	New Guinea	BMNH 1953.17.52	study skin	37	161	462
Porzana tabuensis 4	Tahiti	MNHN CG1973 # 515	study skin	37	161	0
Porzana tabuensis 5	Fiji Islands	BMNH 89.11.3.353	study skin	37	161	0
Porzana tabuensis 6	Philippines	USNM 189871	study skin	37	161	0
Porzana monasa	Kosrae Island	ZISP 138165	study skin	0	157	122
Porzana atra	Henderson I.	BMNH 1913.3.4.11	study skin	37	161	462
Poliolimnas flaviventer	Panama	USNM 460578	study skin	37	161	312
Poliolimnas cinereus	Yap Island	BYUH 2056	study skin	159	161	348
Aenigmatolimnas marginalis	Ghana	USNM462655	study skin	37	161	236
Neocrex colombianus	Ecuador	ANSP 185051	study skin	159	161	579