

SUBSPECIFIC SONG VARIATION AND HYBRID BREAKDOWN AS A REPRODUCTIVE
BARRIER IN *POEPHILA ACUTICAUDA*

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Abstract

The subspecies of *Poephila acuticauda* diverged roughly 0.5 MYA due to a temporary biogeographical barrier. Today, *Poephila acuticauda hecki* inhabits the eastern extreme of the species range in northern Australia and *Poephila acuticauda acuticauda* the western portion with a series of hybrid contact zones in the center. Phenotypic traits such as bill-color and sperm morphology are currently under investigation as potential reproductive barriers to determine whether speciation is still occurring. Because song is a precopulatory behavior in *P. acuticauda*, it is a candidate for both a pre- and post-zygotic reproductive barrier. The former would be facilitated by a clear subspecific difference in song and the latter by F1 hybrids either having similarities to parental lineage songs and reinforcing the subspecific divide in subsequent generations or reductions in song performance indicating hybrid breakdown. Here, we measure a suite of acoustic parameters in Raven Pro as well as frequency excursion and stereotypy to probe for differences in song characteristics and performance between subspecies and F1 hybrids. Principal component analyses revealed differences between the pure subspecies but grouped F1 hybrid song with *P. a. hecki* song. Both stereotypy scores and frequency excursion values were significantly higher in *P. a. acuticauda* song, with no significant difference between *P. a. hecki* and F1 hybrid song. Stereotypy scores were significantly higher for F1 hybrids with *P. a. acuticauda* fathers compared to those with *P. a. hecki* fathers. These data suggest that song could function as a prezygotic barrier via *P. a. acuticauda* song being acoustically different and more complex, thereby facilitating assortative mate choice. In turn, because F1 hybrids with physiological and vocal development deficits may struggle to learn the more complex song, hybrids may then struggle to obtain or breed with a mate making it possible for song to also function as a postzygotic barrier to gene flow.

Introduction

The Long-tailed Finch (*Poephila acuticauda*) is a small grassfinch (*Estrilidae*) endemic to tropical northern Australia. Two subspecies are recognized. The nominate, *P. a. acuticauda*, occurs in the western portion of the range and can generally be distinguished by a yellow bill, while *P. a. hecki* occurs in the eastern portion and is distinguishable by a red bill (Griffith and Hooper, 2017) (Fig. 1). Where the two bill colors meet, around Katherine in the Northern Territory, orange billed individuals can be found (Griffith and Hooper 2017). The two subspecies are about 0.5 MY divergent (Lopez et al., in press), and where the ranges of the two meet they form a hybrid zone about 150 km wide, but the genetic cline and the bill color cline are displaced by about 350 km (Hooper et al., 2019) (Fig. 1).

The evolutionary history of this species is likely to have been shaped by Australia's climate history. Historical aridity has contracted and expanded the range of the long-tailed finch and its evolutionary ancestors, creating temporary biogeographical barriers and leading to both speciation and subspeciation (Rollins et al., 2012). 1.64 million years ago, a geographical barrier led to the divergence of *P. acuticauda* from the Black-throated Finch (*Poephila cincta*). 0.5 million years ago, an arid intrusion along the eastern edge of the Kimberly Plateau that likely divided the Arnhem Land from the Kimberly Plateau facilitated allopatric subspeciation of *P. a. acuticauda* and *P. a. hecki*. Today, the presence of the two distinct subspecies in the east and west, as well as a central contact zone along the eastern edge of the Kimberley Plateau, are supported by mtDNA data (Griffith, S. C., unpublished data). The question remains whether parapatric speciation is still progressing or if the subspecies are reconverging through hybrid backcrossing and introgression.

Recent studies have looked into potential reproductive barriers. Rowe et al. (2015) found small but significant differences in sperm morphology; however, because there is low extra-pair paternity and therefore low sperm competition in this species, the difference in morphology may be due more to genetic drift and a possible bottleneck effect from the low founding population (Rollins et al., 2012) for *P. a. acuticauda* rather than active selective pressure. Van Rooij and Griffith (2012) found no assortative mating associated with bill color, suggesting it may not be a reproductive barrier. However, because the bill color cline is further east than the genetic hybrid zone where this study sampled from, assortative mating between the extremes of bill color cannot be discounted. Evidence is emerging that assortative preference may be at play between captive birds of pure *P. a. acuticauda* and *P. a. hecki* (Griffith, S. C., unpublished data).

Because wild hybrids do occur (Hooper et al., 2019; Lopez et al., in press), for parapatric speciation to occur something else would have to be at play alongside these potential prezygotic barriers. The separation of hybrid zones fills in the picture somewhat by separating where different barriers are facilitated. If sperm morphology only functions at the genetic cline, and assortative mating around bill color at the bill color cline, then their combined power is reduced. An additional mechanism for continued speciation may be hybrid breakdown. Reduction in genetically and/or culturally transmitted traits could act as a postzygotic barrier and prevent F2 hybrids or backcrossing. However, hybridization of traits can also facilitate species convergence if the hybrid phenotype is intermediate or beneficial somehow (Secondi et al., 2011). In the case of *P. acuticauda*, hybrid phenotypes are relatively understudied.

A phenotypic trait that could be either a prezygotic or postzygotic mechanism for reproductive isolation of incipient species is song. Especially in species where song plays a role in copulation and could facilitate assortative mating, it can be a strong behavioral reproductive

barrier. Because of the role of cultural transmission in its development, song is also rapidly evolving and, in cases of complete isolation, often clearly mirrors species and subspecies boundaries (Price, 2008). The result is less clear when isolation is incomplete and populations hybridize. There are also many genetic, physiological, and anatomical components to song as well as learned aspects (Scharff and Adam, 2013; Whiten, 2017) that affect hybrid phenotypes, the outcomes of which could have different possible effects on the populations.

P. acuticauda song is relatively understudied compared to other *estrildids* such as the Zebra Finch (*Taeniopygia guttata*) and has not been explored thoroughly as a possible reproductive barrier. Males typically have one unique song with high observed stereotypy (Zann, 1976) that is sung just before copulation (Zann, 1977). It is not sung as frequently or broadcast as loudly as in other *estrildids*, so the degree to which it affects mate choice may be less, but this requires more research.

One study (Zann, 1976) did compare songs between the two subspecies and found them to be distinct in both the sequence and presence of different elements. However, the sample size for this study was extremely small, using only six *P. a. acuticauda* males and seven *P. a. hecki* males. Further, Zann collected some of these individuals from locations close to the hybrid zones, and without advanced genetic techniques to identify each subspecies or knowledge of the mismatch between bill color and genetics, we cannot be certain of the genetic make-up or song-learning history of the individuals used in the study. Finally, Zann did not include any documented hybrid individuals in his study. Because we know that wild hybrids occur (Hooper et al., 2019; Lopez et al., in press), it is pertinent to explore song as a potential postzygotic reproductive barrier by including the song of hybrid individuals in further analyses. This study is due for replication and expansion.

Today, there are more precise tools available to analyze birdsong. Bioacoustics are difficult to study the evolution of because they leave no physical evidence. This leaves researchers having to make subjective judgements about the similarity of different elements of song. But recently, with the development of Raven Pro (Center for Conservation Bioacoustics, 2019), warbleR (Araya-Salas and Smith-Viadurre, 2017), and frequency excursion software (McClure, 2017; Podos et al., 2016), it has become easier to extract acoustic parameters and make use of data analytics techniques to remove some of the subjectivity involved. There are still concepts that don't lend easily to quantitative measurements such as song complexity (Benedict and Najar, 2019), but they can be approximated and compared between studies using consistent methods. With these tools, there is an opportunity to develop novel methodology for quantifying birdsong.

In this study we compare the songs of *P. a. hecki* and *P. a. acuticauda* males using a combination of Raven Pro and warbleR to detect any distinct subspecific differences in element types, stereotypy, frequency modulation, or other acoustic parameters. We then compare songs from these 'pure' males with the songs from F1 hybrids to test for similarities to parental lineage songs as well as any reductions in complexity and/or performance level that might indicate hybrid breakdown.

Methods

Study Species

Long-tailed Finches are abundant in savanna woodland habitats, occurring in pairs, family groups, and sometimes larger flocks (Zann, 1977). They breed in loose colonies and have high mate-fidelity during and between breeding seasons (van Rooij and Griffith, 2011). Adults have 14-15 distinct calls and one unique song per male that is performed quietly before copulation (Zann, 1977). The identifiable characteristics of the species include brightly colored red, yellow, or orange bills, black throat-patches, and long tails. They exhibit slight sexual dimorphism in their coloring, size of their throat-patch, and length of their tail streamers (van Rooij and Griffith, 2010). They are classified as least conservation concern (BirdLife International, 2021).

Aviary Conditions and Wild Captures

At the time of recording, birds were housed in outdoor aviaries at Macquarie University in Sydney, Australia. For *P. a. hecki* (N = 36), we sampled a captive population bred from birds captured from a breeding population in October Creek, Northern Territory (Rollins et al., 2012; 16°37'S, 134°51'E) in October 2010 (Fig. 1). Our sample included 4 of the original captures, with the other 31 being captive-bred within 5 generations of the original wild individuals. For *P. a. acuticauda*, all birds were 1-5 generations away from the wild-caught birds (N = 31), which were originally captured from two sites (Mt House: 17°02'S, 125°35'E and Nelson's hole: 15°49'S, 127°30'E) in Western Australia in September 2009 (Fig. 1). 26 captive-bred F1 hybrids were sampled.

P. a. hecki and *P. a. acuticauda* individuals were bred in large mixed-sex aviaries containing 50-200 birds of their respective subspecies. Food and water were provided *ad lib*.

Birds remained in this setting through the crystallization of their song, so while they may have heard song from other species and subspecies in adjacent aviaries, they were only ever in physical contact with their own subspecies during this period. In Zebra Finches (*Taeniopygia guttata*), a closely related species, males learn songs only from tutors that they can physically interact with (Naguib, M., unpublished data). Hybrid individuals were bred in small aviaries containing one parent from each subspecies and their siblings. They remained in this set-up where they primarily heard the song of their father through the crystallization of their song. After crystallization, hybrids were transferred to larger mixed-sex aviaries with other hybrids.

Song Recording

At least 3 days before being recorded, males were moved from large outdoor aviaries into indoor cages where they were kept with other males of their subspecies. To record males, they were brought to a semi-outdoor row of aviaries with a wire mesh roof allowing for sunlight and ambient noise that were relatively secluded from other finch aviaries. Each aviary was about 1x3x3 meters and was set up with food and water, 3-5 perches, and a nest box and wire mesh box (from a previous study). Only two aviaries about 10 meters apart were used for each trial. In each aviary, one male was left with one female and a recording device for one hour. For some of the hybrid recording sessions, males were left alone for a period before females were added. Some males did sing while alone, but for this study only recordings of directed song were analyzed. Four females of each subspecies were used in a rotation throughout the day with whichever subspecies was being recorded. Olympus DM-720 recorders were used on recording level *high*, with PCM set at 44.1kHz/16bit. Recorders were connected to Audio-Technica lapel microphones that were clipped to the front of each aviary during trials. After trials, recordings were uploaded to Audacity to have phrases of song visually selected and saved as new wav files.

Recordings from 24 hybrid males, 35 *P. a. hecki* males, and 31 *P. a. acuticauda* males were used for analysis.

Element Classification and Sorting

The initial sorting of elements was done manually. Spectrograms of all *P. a. hecki* and *P. a. acuticauda* included in the study were printed out and cut into individual elements. One phrase per individual was included. Elements were each labeled with band number and subspecies on the back, but then they were sorted into groups of similar looking elements with the researcher blind to both the subspecies and band number. Once elements were sorted, the number of elements occurring in the phrase of each individual and their subspecies identity was recorded.

The initial manual sorting resulted in 8 different element types (Fig. 2A). The number of elements in the phrase of each individual was included as a variable in PCAs comparing phrases from *P. a. hecki* and *P. a. acuticauda*. To analyze the acoustic differences between all the elements and evaluate the initial grouping of them, acoustic parameters were extracted using Raven Pro (see below), and a PCA with individual elements at data points was run to see if the resulting groupings corresponded with the manual groupings. Elements A and C separated out distinctly, while the other elements had a lot of overlap (Fig. 3A). Based on this PCA, and in an attempt to combine similar-looking elements, we created two alternative element groupings to use moving forward with other PCAs comparing the subspecies as well as the hybrids. In the first alternative grouping, elements E, H, and D were combined into a new element “I”, and elements G, F, and B were combined into a new element “J” (Fig. 3B). This grouping is the ACIJ grouping. In the second alternative grouping, elements E and H were combined into a new element “K” and elements G, F, B, and D were combined into a new element “L”. This second alternative is the ACKL grouping. Because the no element, A-H, ACIJ, and ACKL PCAs all

turned out very similar, only two versions of each PCA (no elements and ACIJ) are included in the figures for this paper. The other versions of each PCA plot can be found in the supplementary data (Supplementary Fig. 1).

Element and Song Selection in Raven

To extract acoustic parameters, one representative song from each individual was imported into Raven Pro (Center for Conservation Bioacoustics, 2019). The acoustic parameters included were average entropy (bits), peak frequency contour min frequency (Hz), peak frequency contour max frequency (Hz), average power density (dB FS), delta time (s), peak frequency contour average slope (Hz/ms), delta frequency (Hz), begin time (s), and number of inflection points (Table 1). In addition, the numbers of each element were added in as variables and input manually. When selecting elements in Raven Pro, brightness was set at 60, contrast at 70, the spectrogram was grayscale, and all other settings were left on their defaults. Selections were made starting from the bottom/peak frequency up to the fourth harmonic layer or the last clearly visible layer of the element reaching the highest frequency if less than four layers were visible. The entire passage was also selected to be inputted into PCAs and spectrogram cross correlations with the associated acoustic parameters and element counts.

Principal Component Analyses to Compare Subspecies and F1 Hybrids

Selections and acoustic parameters were exported from Raven into a data frame in R. From there, the data table was manipulated to regroup elements into all three element combinations (A-H, ACIJ, and ACKL) as well as one with no element counts. Data frames were reduced to only entire phrases selected from Raven. With both element counts and acoustic parameters as variables, four versions of a PCA with representative phrases from *P. a. hecki* and

P. a. acuticauda were produced, and four versions of a PCA with representative phrases from *P. a. hecki*, *P. a. acuticauda*, and F1 hybrids split by father subspecies were produced using the *prcomp* function in the *stats* R package. Principal component values were viewed using the *summary* and *print* functions in *base* R. PCA plots were visualized in 2D using the *fviz_pca_ind* function in the *factoextra* R package and as 3D interactive HTML pages using the *plot_ly* function in the *plotly* R package.

Stereotypy Indices

Stereotypy indices were calculated for each individual that had ten or more viable phrases recorded. Six *P. a. acuticauda*, seven *P. a. hecki*, and three hybrid individuals did not meet this criterion, leaving the new sample sizes at 29 *P. a. acuticauda*, 24 *P. a. hecki*, and 21 hybrid individuals. Recordings were imported into Raven, where 10 complete phrases were selected up to the highest visible harmonic or the fourth layer with brightness at 60 and contrast at 70 on a grayscale spectrogram. Phrases were imported into R and separated into selection tables for each individual containing their 10 phrases. Comparison matrices were constructed for each individual so that each phrase was compared to all 9 other phrases but not to itself. Comparison matrices were then put into the *xcorr* function in the *warbleR* package, producing a correlation value for each comparison. The average of those correlation values was calculated as the stereotypy score for each individual. These scores were run through an ANOVA, and then post-hoc Tukey's tests to investigate differences between *P. a. acuticauda* and *P. a. hecki*, between *P. a. acuticauda* and hybrids, and between *P. a. hecki* and hybrids. A two-sample t-test was used to compare hybrids split by father subspecies. These indices were calculated both to confirm the previous finding (Zann, 1976) that the use of a single phrase was representative of that individual as well as to be a measure of performance ability and song development. Use of a stereotypy index as a

performance proxy is an emerging method in birdsong analysis (Holveck and Riebel, 2007; Leadbeater et al., 2005; Scharff and Adam, 1991; Thompson et al., 2013; Zann, 1976) due to the relationship between song crystallization and vocal development (Thorpe, 1958).

Frequency Excursion Analysis

Frequency excursion (FEX) is a sum of both within and between-note frequency modulations on a per-time basis. It is a measure of vocal performance that accounts for subtle variations in performance overlooked by measures like vocal deviation (Podos et al., 2016). Here, we used the updated frequency excursion calculator TweetyPy run in Unix on a Mac (McClure, 2017). We used the erase tool to clean up background noise around notes and between harmonic layers. Buzz notes were left alone. After erasing was finished, the program provided an FEX value, which was recorded. One representative phrase per individual was measured, giving us one FEX value per individual. These values were then run through an ANOVA, and then post-hoc Tukey's tests to investigate differences between *P. a. acuticauda* and *P. a. hecki*, between *P. a. acuticauda* and hybrids, and between *P. a. hecki* and hybrids. A two-sample t-test was used to compare hybrids split by father subspecies.

Eliminating Age as a Confound

Both stereotypy and performance ability increase with age during song development (Thorpe, 1958). Further, the age of song crystallization for *P. a. acuticauda* is unknown and can only be estimated from the value of 80-100 days known for the Zebra Finch (*Taeniopygia guttata*) (Böhner, 1990). Therefore, even though the youngest bird in our study was recorded at 144 days and the ages for hybrids had an age range of 256 days above this minimum, we still checked for any relationship between age and either stereotypy scores or FEX scores to ensure

they weren't impacted by developmental factors. To do this, correlations were run between age and stereotypy indices ($r = 0.23$) as well as between age and FEX scores ($r = -0.08$) for hybrids (Supplementary Fig. 2A-B). Further, hybrids were recorded again about 90 days later, and these new recordings were put into a PCA with the pure subspecies. The new recordings grouped similarly to the original hybrid recordings (Supplementary Fig. 2A).

Father/son comparisons

A small number of father-son pairs were serendipitously included in this study. To follow up with previous comparisons between groups of hybrids and the songs of their father subspecies, we also ran spectrogram cross correlations between the phrases of these individual fathers and sons and tallied the element types appearing in each song to be compared. The sample size was extremely small for this, with only four hybrids from two *P. a. hecki* fathers and one hybrid from a *P. a. acuticauda* father.

Results

Element Categorization

Initial manual sorting of *P. a. hecki* and *P. a. acuticauda* elements while blind to subspecies resulted in eight different element types, A-H (Fig. 2A). Elements D and H only showed up in *P. a. acuticauda* song, E, F, and G only appeared in *P. a. hecki* song, and A, B, and C appeared in both, although there was only one instance of element A in *P. a. hecki*, which was much quieter and less distinct than any occurrence in *P. a. acuticauda*. Principal component analyses for elements B and C with acoustic parameters as inputs revealed a clear separation of *P. a. acuticauda* element C from *P. a. hecki* element C (Supplementary Fig. 3B), but only a slight gradation of element B between the two subspecies (Supplementary Fig. 3A). When all elements were put into a principal component analysis together with acoustic parameters as input, elements A and C formed distinct groups along both the first and second principal components. Elements B and D-H clustered together but formed some more distinct regions coinciding with our element categories from the manual sorting when visualized with the first three principal components. A and C appear to be the only elements from the blind sorting that are quantitatively distinct. The rest of the elements overlap with each other and, aside from B, only show up in one subspecies, meaning that they could be different variants of the same element (Fig. 3; Table 2). Concrete categorizations like this cannot be made without an evolutionary history of the elements, which is currently unavailable.

Principal Component Analyses Comparing *P. a. hecki*, *P. a. acuticauda*, and F1 Hybrids

Principal component analyses run with only presence/absence of element types for each individual song and with continuous counts of element occurrences in each individual song as inputs both grouped individuals by subspecies (Supplementary Fig. 4). With only acoustic

parameters as inputs (Table 1), separation of subspecies was also distinct (Fig. 4). Individuals were separated clearly by the first two principal components with the highest contributing variables being PFC minimum frequency, delta time, delta frequency, and inflection points (Table 3). Principal component analyses with reduced ACIJ groupings as well as acoustic parameter inputs also separated individuals based on subspecies along the first two principal components (Fig. 4). Here, the frequency of elements I, A, and C were important variables (Table 3).

The same inputs to principal component analyses with representative songs from all *P. a. hecki*, *P. a. acuticauda*, and F1 hybrid individuals split by father subspecies revealed a continued separation of *P. a. hecki*, *P. a. acuticauda* along the first two principal components with the same variables contributing the most (Table 3), but no clear separation of the hybrids based on father subspecies (Fig. 4). The majority of hybrid data points overlapped with *P. a. hecki*, with a few seeping into the *P. a. acuticauda* grouping. The hybrid points are somewhat separated from *P. a. hecki* data points along PC2 in the plot with no element counts, and when reduced IJ element counts are added in, they are separated from *P. a. hecki* points along both PC1 and PC2 (Fig. 4).

Stereotypy Indices

The ANOVA of stereotypy indices between *P. a. acuticauda*, *P. a. hecki*, and hybrids was significant ($p < 0.0001$). Post-hoc Tukey's tests revealed that stereotypy indices for *P. a. acuticauda* (mean = 0.692) were significantly higher than *P. a. hecki* (mean = 0.611, $p < 0.0001$), and hybrids (mean = 0.608, $p < 0.0001$). Indices for *P. a. hecki* and hybrids were not significantly different ($p = 0.983$). Stereotypy indices for hybrids with *P. a. acuticauda* fathers (mean = 0.649) were significantly higher than hybrids with *P. a. hecki* fathers (mean = 0.567, $p <$

0.001). There was no relationship between age when recorded and stereotypy indices within hybrids ($r = 0.23$) (Table 4).

Frequency Excursion Analysis

The ANOVA of frequency excursion scores between *P. a. acuticauda*, *P. a. hecki*, and hybrids was significant ($p < 0.0001$). Post-hoc Tukey's tests revealed that FEX scores for *P. a. acuticauda* (mean = 34.64) were significantly higher than *P. a. hecki* (mean = 18.11, $p < 0.0001$) and hybrids (mean = 22.21, $p < 0.001$). Scores for *P. a. hecki* and hybrids were not significantly different ($p = 0.169$). FEX scores for hybrids with *P. a. acuticauda* fathers (mean = 24.42) were higher than hybrids with *P. a. hecki* fathers (mean = 19.2) but not significantly ($p = 0.105$). There was no relationship between age when recorded and FEX scores within hybrids ($r = -0.08$) (Table 4).

Father/Son Comparisons

The average spectrogram cross correlation value between *P. a. hecki* and hybrid sons was 0.482, and the correlation between the only pair of a *P. a. acuticauda* father and a hybrid son was 0.549 (Fig. 5). In the *P. a. hecki* and hybrid pairs, most elements appeared to be conserved in hybrid song. In the songs of W1406 and W1407, elements appear slightly distorted in hybrid song (Supplementary Fig. 5). In the *P. a. acuticauda* and a hybrid pair, element D appears distorted, and element A is not conserved in the hybrid song (Fig. 5).

Discussion

This study presents evidence that the song of the two subspecies of *P. acuticauda* are distinct in both acoustic structure as well as performance level, with that of *P. a. acuticauda* having both significantly higher stereotypy scores and frequency excursion values. Further, the song of F1 hybrids has a similar structural and performance profile to that of *P. a. hecki* regardless of parental lineage. However, when stereotypy scores are compared between hybrids split by parental lineage, those with *P. a. acuticauda* fathers had significantly higher stereotypy scores. The same comparison for FEX scores showed no significant difference.

The difference between the song of *P. a. acuticauda* and *P. a. hecki* observed here confirms the conclusion of Zann (1976) with a larger sample size, a higher confidence level of subspecies identity, and more sophisticated modern software techniques to categorize song elements and quantify structure. Our methods still required some subjective categorizations of elements and can be improved upon using machine learning techniques, but quantitative acoustic measurements were the main criteria used to identify song components.

Like Zann (1976), we also found differences in the types and number of elements. The major elements distinguishing the two subspecies in our study were A (the buzz note, Fig. 2) and C (the intro notes, Fig. 2). Zann (1976) also focused on the difference in the number of intro notes (C), and briefly noted the buzz note, labeled K in his study and appearing only in the song of *P. a. hecki*. This occurrence of the buzz note was the opposite of our findings, where it occurred primarily in *P. a. acuticauda*.

Two hypotheses might explain this mismatch in which subspecies the buzz note occurred. First, the *P. a. hecki* individuals (N=3) that sang the buzz note in Zann's study were captured very close to the bill color hybrid zone, leaving open the possibility that they might not have

been genetically *P. a. hecki* but rather some type of hybrid or backcrossed individuals. Second, because song evolves so quickly and can vary greatly by location, it is possible that our study and Zann's study just captured different local dialects, or that song changed and the buzz element drifted across the hybrid zone in the 45-year gap between these two studies. An understanding of song variation across the entire range of *P. acuticauda* as well as a longitudinal record of the change in local dialects are necessary to further explore this hypothesis.

Regardless of the specific distinguishing characteristics, because of the structural and complexity differences observed here between the two subspecies, song-based mate choice is a candidate prezygotic reproductive barrier maintaining parapatric speciation between pure *P. a. acuticauda* and *P. a. hecki* individuals. However, more research is required on the degree of assortative mating associated with song to confirm this hypothesis.

Because wild hybrids do occur, and because of the presence of geographically distinct hybrid zones, a comparison between the song of various hybrids, backcrosses, and other combinations of genetic and phenotypic profiles is extremely relevant for insight into song as a postzygotic barrier between incipient species. Our exploration of the song of F1 hybrids is the first for this species. The results of our principal component analyses suggest that F1 hybrid song is acoustically more similar to that of *P. a. hecki* than *P. a. acuticauda* and cannot be easily distinguished based on father subspecies. Further, hybrid song is significantly less stereotyped and exhibits less frequency modulation than the song of *P. a. acuticauda* but is not significantly different in either measure from the song of *P. a. hecki*. These results prompt the hypothesis that the song of *P. a. acuticauda* individuals is more complicated and difficult for hybrid offspring to learn. This fits into a narrative of hybrid breakdown. If the F1 hybrids have reduced genetic predispositions for learning, vocal ability, or other physiological factors of song, which has been

seen in hybrids of other species (McQuillan et al., 2018), they might be struggling to learn a more complicated song, which in turn could lead to reproductive barriers against F2 or backcrossed generations.

This is further supported by the father-son comparison, in which the hybrid offspring of a *P. a. acuticauda* individual was unable to replicate some elements of the song, almost completely dropping the buzz note. Further research into the hybridized genome relating to the neurological song system and vocal development of this species would help explore this hypothesis. Our results are also only applicable to F1 crosses of pure *P. a. hecki* and *P. a. acuticauda* individuals. Studies using backcrossed individuals as well as further generations of hybrids beyond F1 would provide more insight into the degree of hybrid breakdown occurring and how this might play out in the various hybrid contact zones. A follow-up study of female mate choice in regard to hybrid song would also clarify the ability of hybrid breakdown in song to act as a postzygotic barrier.

Additionally, our measures of stereotypy and frequency excursion are only proxies for song complexity and performance ability. Birdsong complexity is a highly subjective concept (Benedict and Najar, 2019) and is difficult to quantify. Our study is just one experimental approach. We based our methods primarily on the stereotypy score in Scharff and Nottebohm (1991) and the frequency excursion analysis in Podos et al. (2016), but both are still just proxies and have little relevance for comparison between studies unless they are applied consistently in future work. More research is required to develop the best methods for quantifying abstract ideas about birdsong with so much new software now at our disposal.

Another significant area for further study is in the classification and evolutionary history of song elements. While both our manual and quantitative approaches identified distinct song

elements, the evolutionary development and relationships between elements remains unclear. Elements from a ‘common ancestor’ and those that converged to a similar structure are currently indistinguishable. Even with machine learning techniques to objectively categorize different elements (Odom et al., 2021), it is still very difficult to trace the evolutionary relationship between song elements because song leaves no fossil record. It would benefit our understanding of the various elements in *P. acuticauda* and broaden their context to collect longitudinal song data, wild recordings from throughout their range, and recordings from evolutionary relatives such as the Black-throated Finch (*Poephila cincta*).

This study provides a new angle to explore in piecing together the evolutionary snapshot of *P. acuticauda* present today. Song often plays a role in reproductive isolation of incipient species, and our study suggests it has potential as both a prezygotic barrier based on the subspecific difference and a postzygotic barrier facilitated by hybrid vocal breakdown. Further, we propose a mechanism for postzygotic isolation via *P. a. acticauda* song being difficult for hybrids to learn due to deterioration of vocal development and production. However, the story is still incomplete. To better understand the variation in song, research is required on the bottleneck effect that may have occurred in *P. a. acuticauda* (Rowe et al., 2015) as well as how their song functions in their life history. Some research suggests that variation is naturally lower in bird species that don’t broadcast their song for territorial purposes (Cate and Slabbekoorn, 2002). This could affect the variation possible in *P. acuticauda*. In the larger story of *P. acuticauda* evolution though, many factors other than song are likely also at play. Sperm morphology is being explored (Rowe et al., 2015), but niche divergence, copulatory behavior, or other behavioral and morphological traits could also play a role. There are likely many factors at play in such a complex series of contact zones.

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Figures

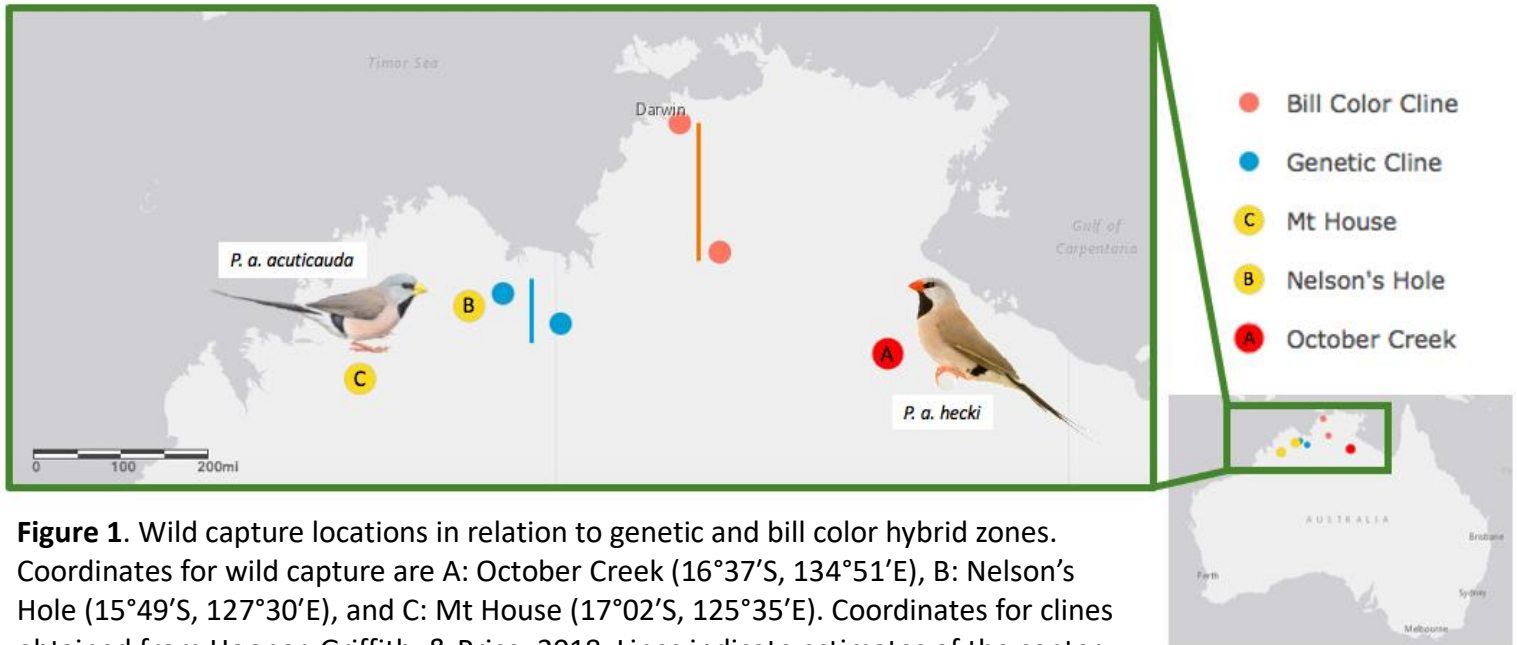


Figure 1. Wild capture locations in relation to genetic and bill color hybrid zones. Coordinates for wild capture are A: October Creek ($16^{\circ}37'S$, $134^{\circ}51'E$), B: Nelson's Hole ($15^{\circ}49'S$, $127^{\circ}30'E$), and C: Mt House ($17^{\circ}02'S$, $125^{\circ}35'E$). Coordinates for clines obtained from Hooper, Griffith, & Price, 2018. Lines indicate estimates of the center of each hybrid zone based on the coordinates of the bounds.

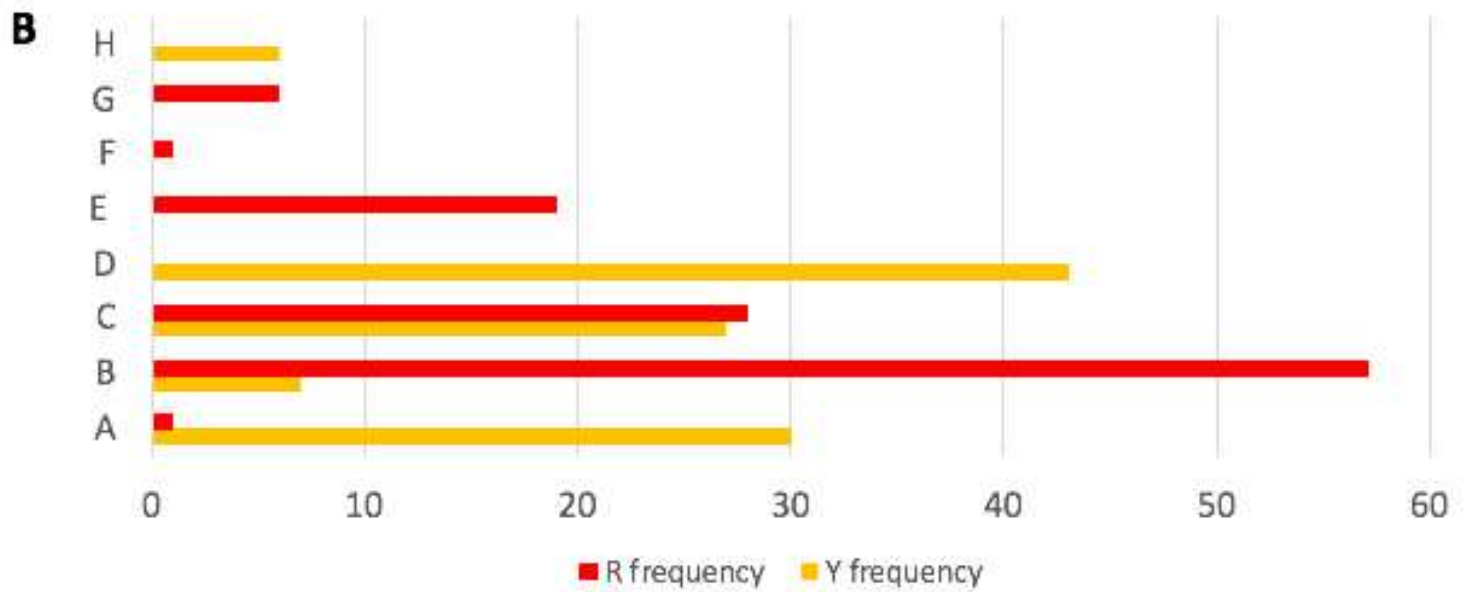
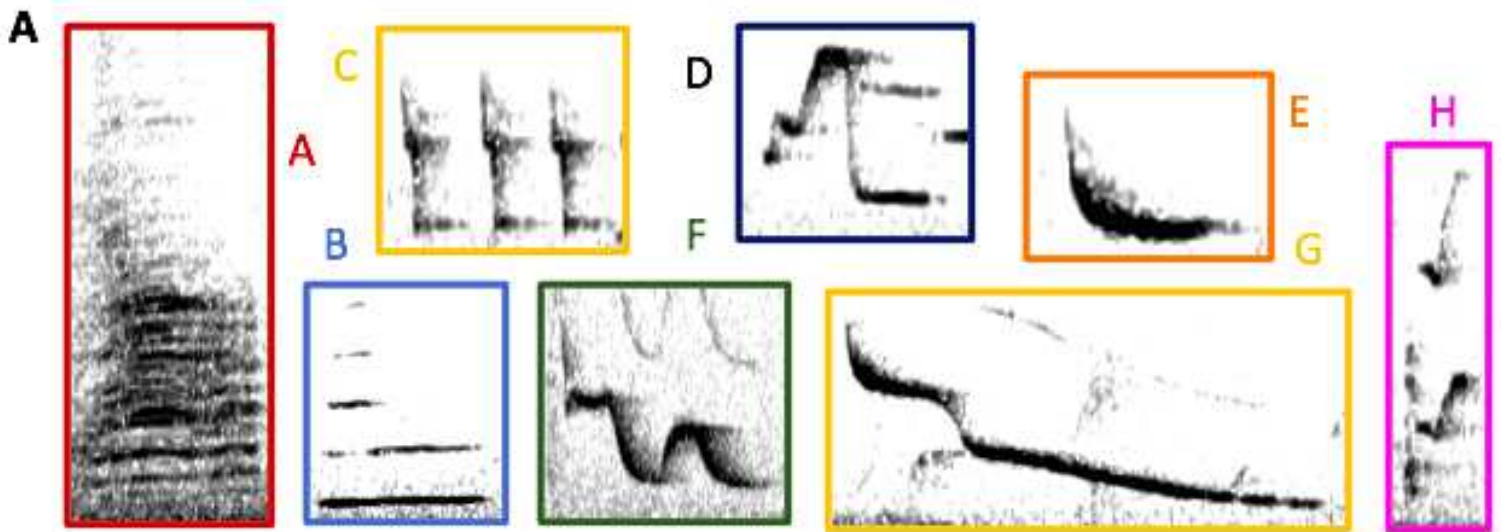
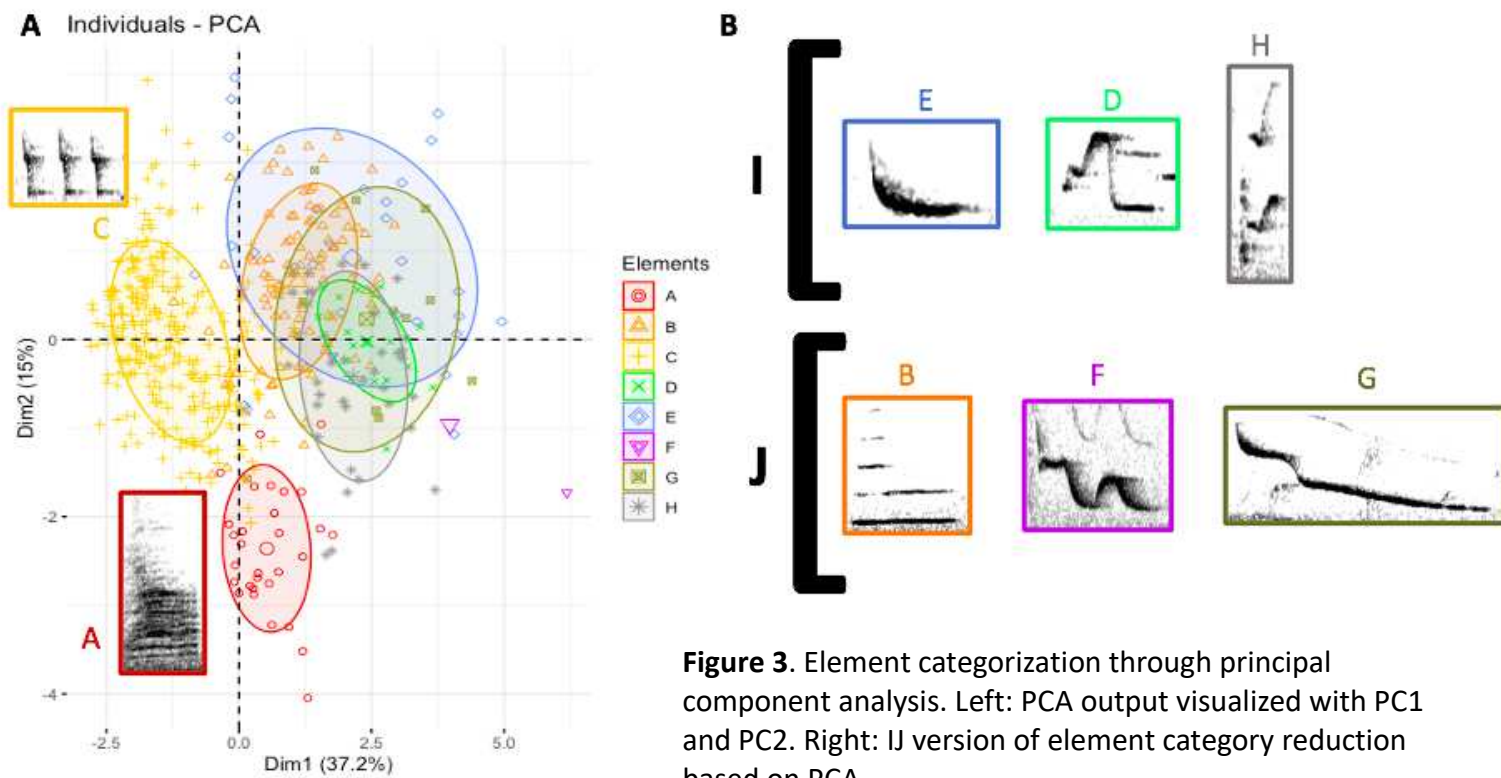


Figure 2. Element classifications from manual blind sorting (top) and frequency of elements in each subspecies (bottom) where R is *P. a. hecki* and Y is *P. a. acuticauda*.



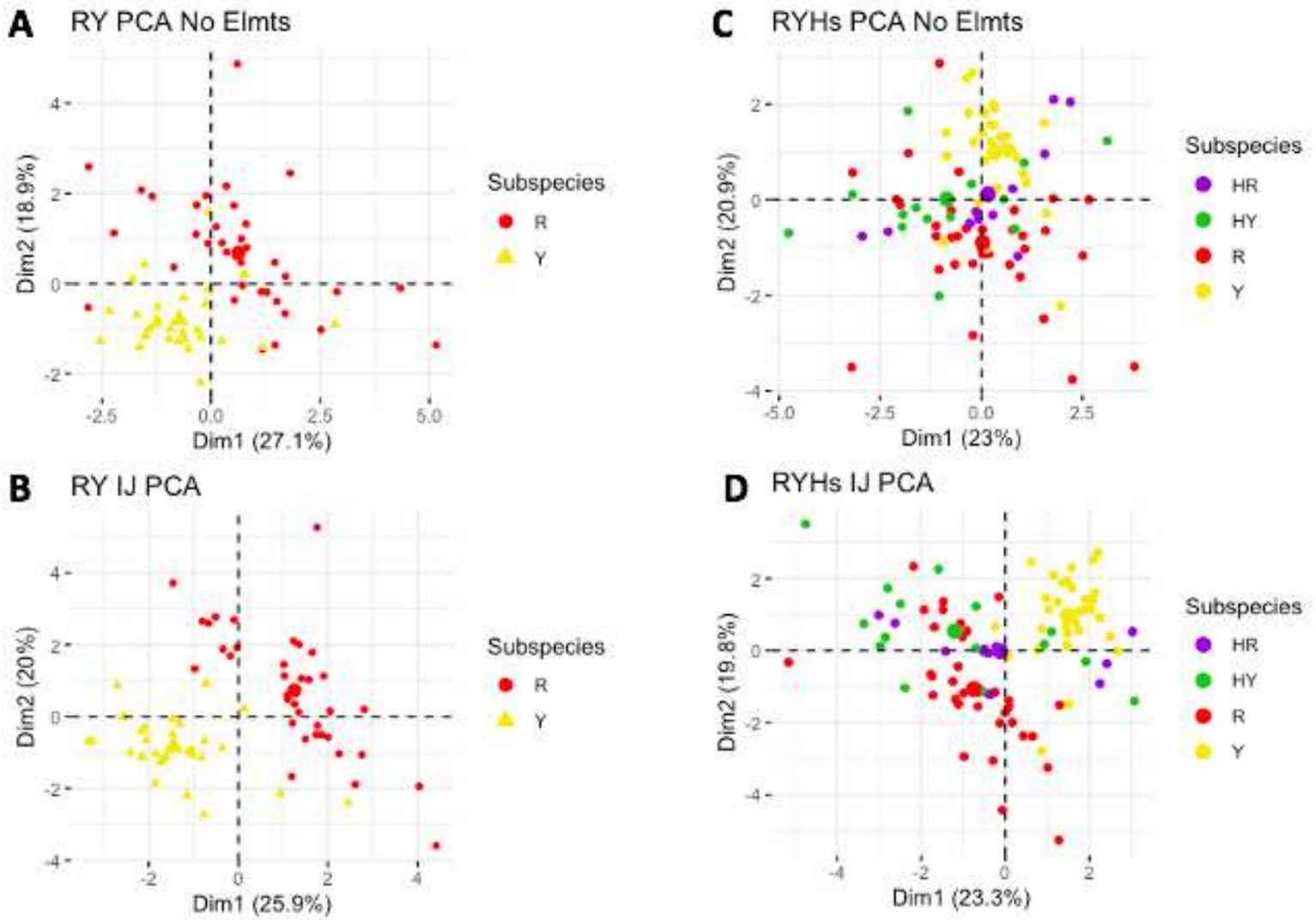
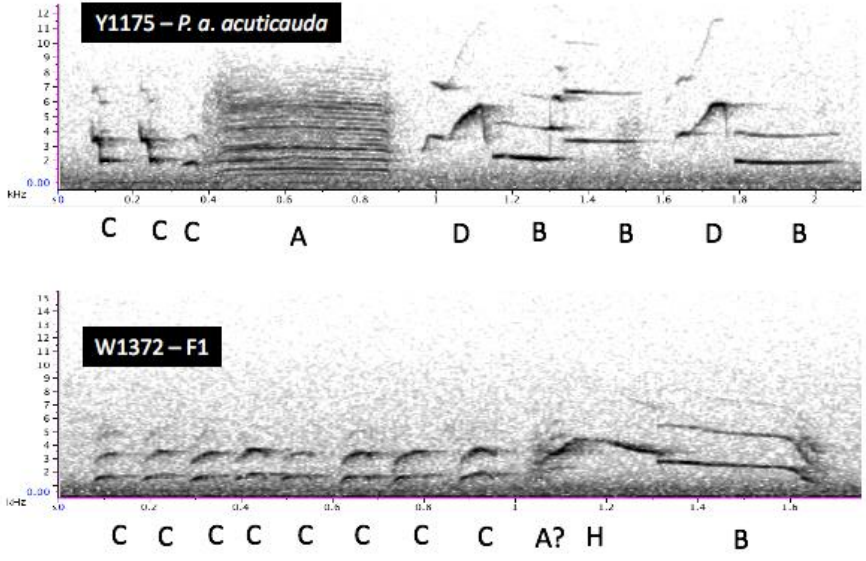


Figure 4. Principle component analyses comparing song based on acoustic parameters and frequency of elements. A) Comparing *P. a. hecki* and *P. a. acuticauda* without element frequencies. B) Comparing *P. a. hecki* and *P. a. acuticauda* with element frequencies based on reduced IJ groupings. C) Comparing *P. a. hecki*, *P. a. acuticauda*, and F1 hybrids grouped by father subspecies without element frequencies. B) Comparing *P. a. hecki*, *P. a. acuticauda*, and F1 hybrids groups by father subspecies with element frequencies based on reduced IJ groupings.



	Father	F1 Son	Correlation
<i>P. a. acuticauda</i>	Y1175	W1372	0.55
<i>P. a. hecki</i>	Y1418	W1406	0.48
	Y1273	W1384	0.59
	Y1273	W1386	0.63
<i>P. a. hecki</i> average			0.48

Figure 5. Direct father-son comparisons. Top left: Raven Pro spectrogram visualization of *P. a. acuticauda* Y1175 father song. Bottom left: Raven Pro spectrogram visualization of F1 hybrid W1372 son song. Right: Correlation values between father-son pairs.

Tables

Table 1. Acoustic Parameters Measured in Raven Pro

Inflection Points

Average Entropy (bits)

Peak Frequency Contour Minimum Frequency (Hz)

Peak Frequency Contour Maximum Frequency (Hz)

Average Power Density (dB FS)

Delta Time (s)

Peak Frequency Contour Average Slope (Hz/ms)

Delta Frequency (Hz)

Start Time (s)

Principal Component	Proportion of Variance	Highest Contributing Acoustic Parameters	Variable Importance
PC1	0.370	Peak Frequency Contour Maximum Frequency (Hz)	0.469
		Delta Time (s)	0.420
		Delta Frequency (Hz)	0.410
PC2	0.161	Average Entropy (Bits)	-0.583
		Inflection Points	-0.484
		Peak Frequency Contour Minimum Frequency (Hz)	0.440

Table 2. Principal component variable importance for element sorting.

PCA	Principal Component	Proportion of Variance	Highest Contributing Acoustic Parameters	Variable Importance
RY No Elements	PC1	0.271	Peak Frequency Contour Maximum Frequency (Hz)	-0.499
			Delta Time (s)	-0.462
			Delta Frequency (Hz)	-0.376
	PC2	0.189	Inflection Points	0.605
			Peak Frequency Contour Minimum Frequency (Hz)	-0.487
			Delta Time (s)	0.466
RY U	PC1	0.259	Frequency of Element I	-0.427
			Peak Frequency Contour Minimum Frequency (Hz)	-0.400
			Frequency of Element A	-0.387
	PC2	0.200	Inflection Points	0.514
			Delta Time (s)	0.487
			Frequency of Element C	0.463
RYHs no Elements	PC1	0.230	Delta Time (s)	-0.653
			Inflection Points	-0.646
			Peak Frequency Contour Minimum Frequency (Hz)	0.258
	PC2	0.209	Peak Frequency Contour Maximum Frequency (Hz)	0.583
			Peak Frequency Contour Minimum Frequency (Hz)	0.527
			Delta Frequency (Hz)	0.366
RYHs U	PC1	0.233	Inflection Points	-0.424
			Peak Frequency Contour Minimum Frequency (Hz)	0.418
			Frequency of Element C	-0.400
	PC2	0.198	Delta Time (s)	0.410
			Frequency of Element I	0.401
			Peak Frequency Contour Maximum Frequency (Hz)	0.393

Table 3. Principal component analysis values for *P. a. hecki*, *P. a. acuticauda*, and F1 hybrid comparisons.

Analysis of Variance and post-hoc Tukey's tests between *P. a. hecki*, *P. a. acuticauda*, and F1 hybrids

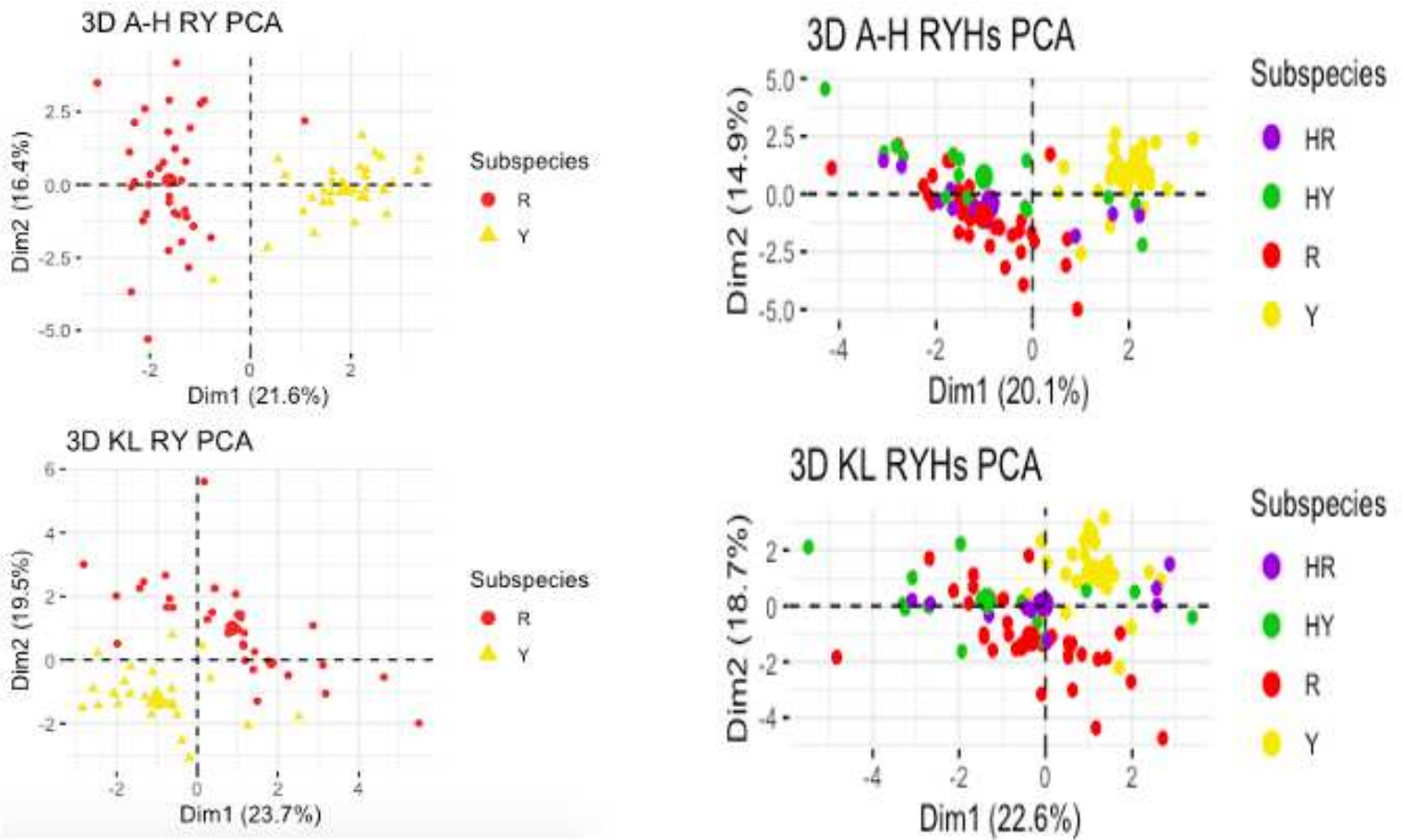
	ANOVA p-value	Contrast	Estimate	p-value
Stereotypy Indices	2.269 x 10 ⁻⁶	H-R	-0.00294	0.9828
		H-Y	-0.08377	< 0.0001
		R-Y	-0.08083	< 0.0001
Frequency Excursion Scores	5.151 x 10 ⁻⁹	H-R	4.1	0.1692
		H-Y	-10.4	0.0001
		R-Y	-14.5	< 0.0001

Two-sample t-test assuming unequal variances between F1 hybrids with *P. a. hecki* and *P. a. acuticauda* fathers

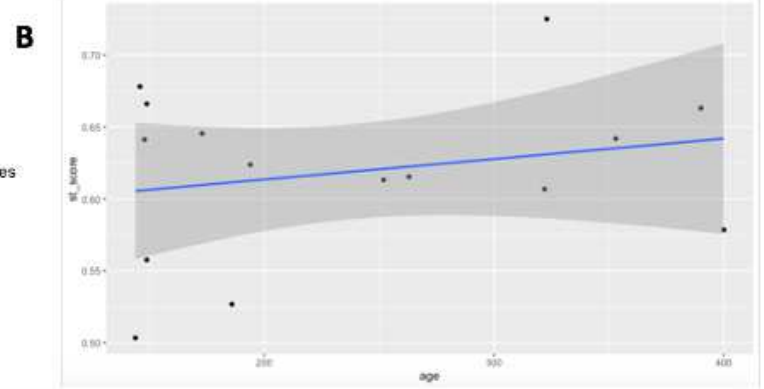
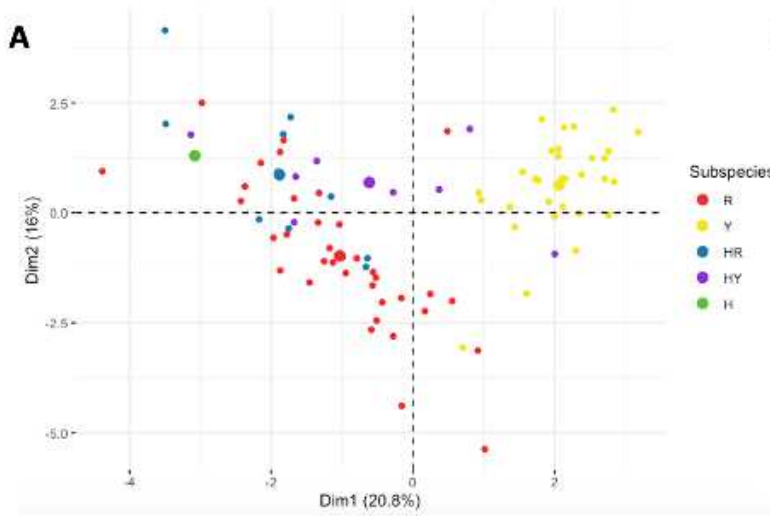
	<i>P. a. hecki</i> Father mean	<i>P. a. acuticauda</i> Father mean	Two-tailed p-value
Stereotypy Indices	0.567	0.649	0.000359
Frequency Excursion Scores	19.2	24.42	0.105

Table 4. Analysis of stereotypy indices and frequency excursion scores between *P. a. hecki*, *P. a. acuticauda*, and F1 hybrids grouped by father subspecies.

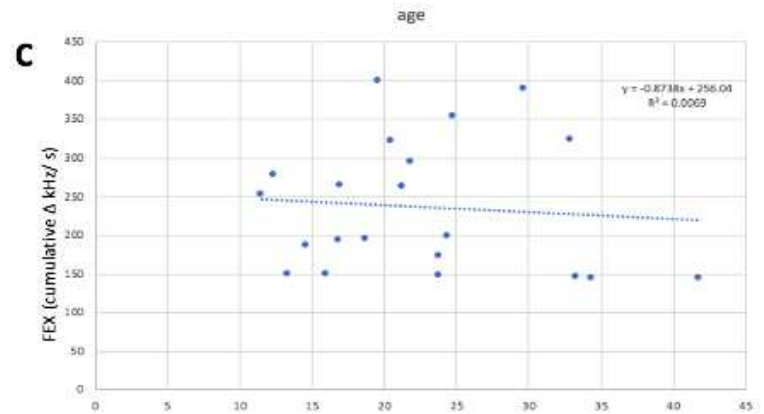
Supplementary Figures

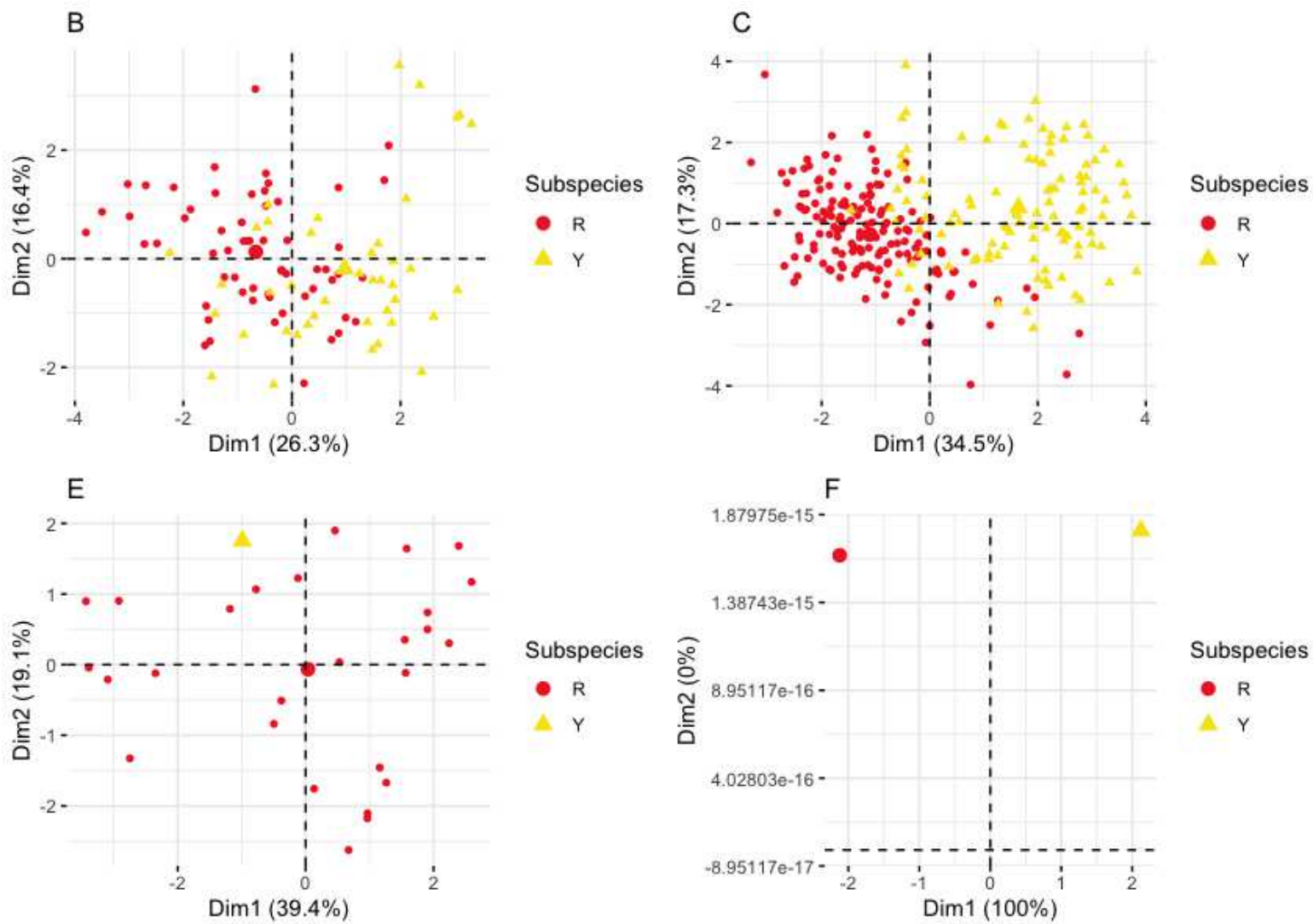


Supplementary Figure 1. Top left: PCA comparing *P. a. acuticauda* and *P. a. hecki* with A-H elements and acoustic parameters as inputs. Bottom left: PCA comparing *P. a. acuticauda* and *P. a. hecki* with reduced ACKL elements and acoustic parameters as inputs. Top right: PCA comparing *P. a. acuticauda*, *P. a. hecki*, and F1 hybrids with A-H elements and acoustic parameters as inputs. Bottom right: PCA comparing *P. a. acuticauda*, *P. a. hecki*, and F1 hybrids with reduced ACKL elements and acoustic parameters as inputs.



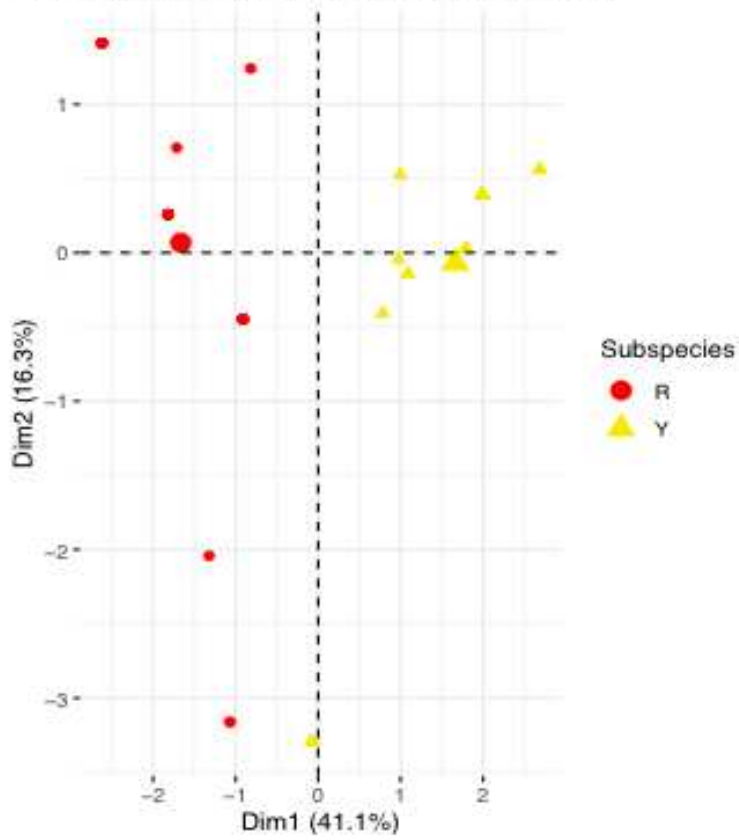
Supplementary Figure 2. Elimination of age as a confound for stereotypy and frequency modulation. A) Principal component analysis with A-H elements and suite of acoustic parameters as inputs. Hybrid recordings are about 90 days later than original recordings. B) Correlation of stereotypy scores against age (days). C) Correlation of frequency excursion values against age (days).



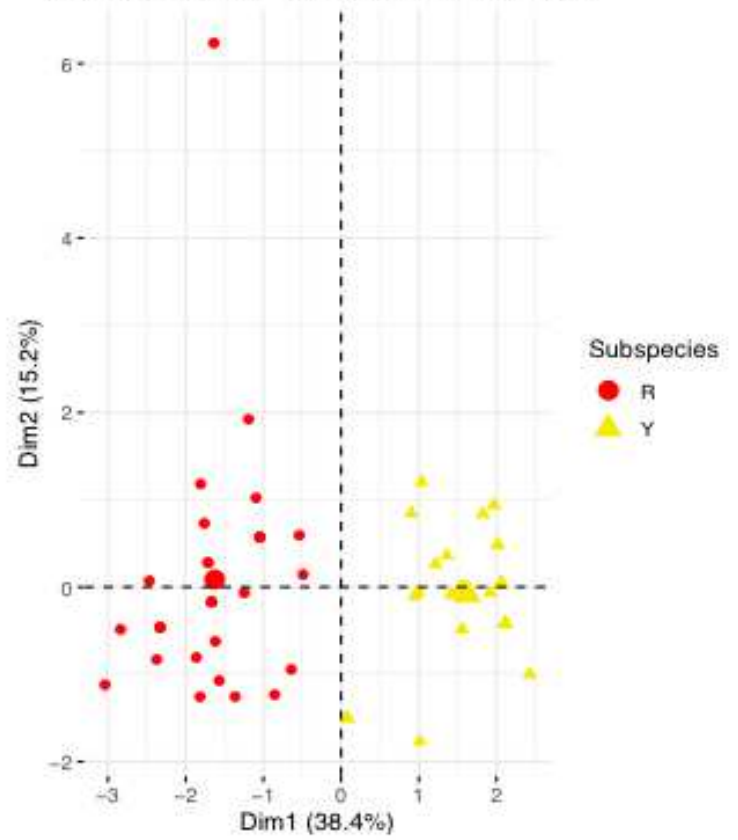


Supplementary Figure 3. Principal component analysis of individual elements compared between *P. a. acuticauda* and *P. a. hecki*.

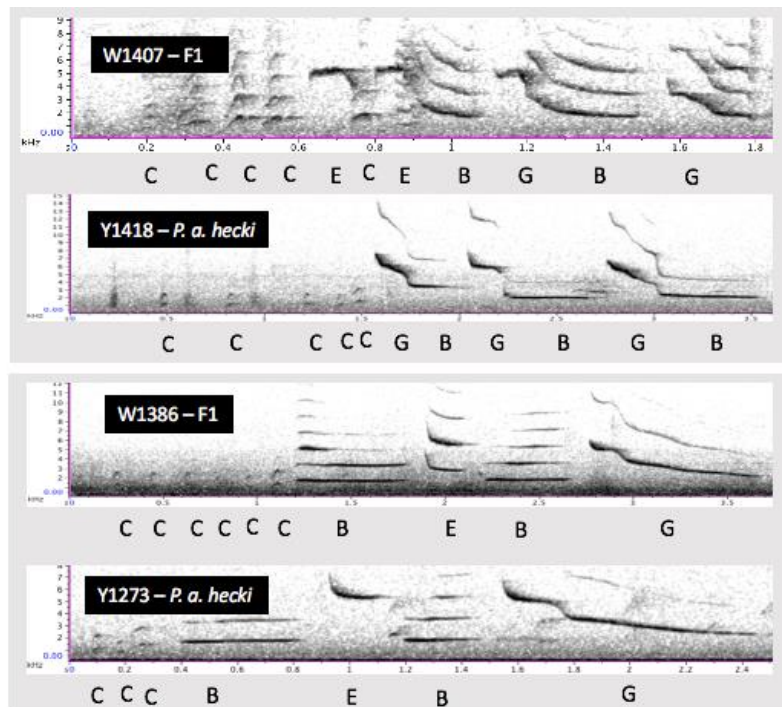
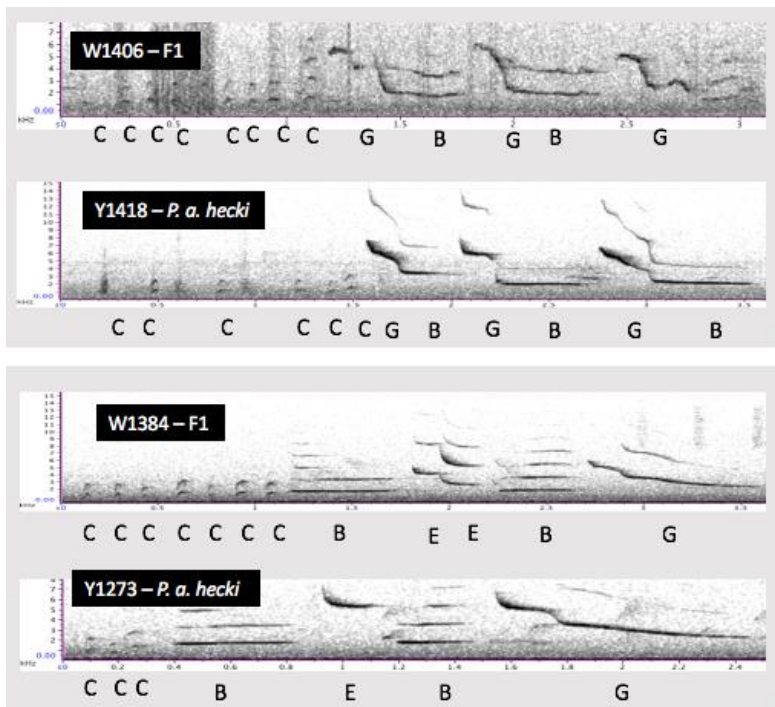
2D PCA-plot from presence-absence data



2D PCA-plot from continuous count data



Supplementary Figure 4. Principal component analyses comparing pure *P. a. acuticauda* and *P. a. hecki* individuals based on only element presence/absence and frequencies.



Supplementary Figure 5. Direct father-son comparisons between *P. a. hecki* and F1 hybrids.

Appendix A

Stereotypy Scores

<i>P. a. hecki</i>		<i>P. a. acuticauda</i>		F1 hybrids	
Band Number	Stereotypy Score	Band Number	Stereotypy Score	Band Number	Stereotypy Score
73783	0.61899764	W1007	0.73192426	W1243	0.66304937
73907	0.54761295	W1008	0.54723723	W1244	0.57860957
73946	0.61267276	W1013	0.69553907	W1284	0.6419571
W1015	0.7161128	W1508	0.52704462	W1285	0.61543317
Y0358	0.68445308	Y0446	0.68296211	W1307	0.72495029
Y0442	0.63660986	Y0634	0.66908371	W1309	0.60691037
Y0509	0.54982964	Y1023	0.70754229	W1312	0.61318618
Y0533	0.64442152	Y1045	0.75361508	W1320	0.54270918
Y0659	0.54596886	Y1059	0.6899461	W1345	0.52689355
Y0714	0.63528689	Y1060	0.70361221	W1365	0.60554274
Y0742	0.64866941	Y1098	0.66760414	W1367	0.6391454
Y0773	0.66152333	Y1106	0.77137766	W1372	0.64541432
Y1042	0.62804218	Y1132	0.72786758	W1394	0.62381643
Y1043	0.59555226	Y1175	0.73487957	W1402	0.66608405
Y1067	0.62689453	Y1176	0.70804715	W1403	0.5576902
Y1128	0.63262899	Y1183	0.76160184	W1404	0.6412569
Y1137	0.6959697	Y1194	0.74384326	W1407	0.50346734
Y1252	0.55261316	Y1382	0.66253475	W1413	0.67808415
Y1253	0.62958012	Y1388	0.65721759	W1416	0.52952116
Y1254	0.58381919	Y1437	0.66586406	W1421	0.55527757
Y1261	0.67129352	Y1509	0.68705614		
Y1271	0.61370888	Y1512	0.72141209		
Y1273	0.53141722				
Y1281	0.54447081				
Y1286	0.59485235				
Y1406	0.50374225				
Y1408	0.58900779				
Y1419	0.6090393				
average	0.61088539		0.69171875		0.60794995

Appendix B

FEX scores (cumulative Δ kHz/ s)			
<i>P. a. hecki</i>	<i>P. a. acuticauda</i>	F1 hybrids	
17.1	32.6	29.7	
4.92	21.9	19.6	
12	25	24.8	
28.6	27.1	21.3	
15.8	34.8	17	
17.3	50.1	32.9	
22.4	34	20.5	
12.7	25.7	11.5	
14.1	53.4	24.8	
22.4	24.6	14.6	
45.5	46.5	24.4	
28.5	22.6	12.4	
9.4	44.3	28.5	
18.5	17.8	15.5	
5.79	43.6	23.8	
14.4	21.2	18.7	
18.7	26.2	21.8	
21	26.3	16.9	
27.9	34.9	16	
18	36.4	13.3	
16.3	41.7	23.8	
17.4	38.4	34.4	
18.5	30.9	41.8	
7.26	39.4	33.3	
29.1	43	13.5	
15.8	34.3	22.7	
24	46.7		
15.6	14.2		
16.1	19.5		
22.1	24.7		
30.7	30.1		
9.49			
19.1			
12.4			
7.26			
15.9			
average	18.1116667	32.6419355	22.2115385