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Geographic Patterns in Chiffchaff Vocalizations: A Multi-Subspecies Analysis Across Hybrid Zones

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ABSTRACT

Bird song serves as a critical medium for intraspecific communication, playing indispensable roles in mate attraction, territorial defense, and species recognition. As a complex learned behavior, bird song often exhibits significant geographical variation, which can be influenced by environmental factors, cultural transmission, and genetic divergence. The Common Chiffchaff (Phylloscopus collybita) complex, a widespread Palearctic passerine, is a renowned model system for studying evolutionary processes, particularly due to its intricate pattern of subspecies distribution and the existence of secondary contact zones where hybridization occurs. This study meticulously investigates the geographical variation in the song characteristics of three key chiffchaff subspecies: P. c. collybita (Western European), P. c. abietinus (Eastern European), and P. c. tristis (Siberian), focusing specifically on their distinct core ranges and two prominent secondary contact zones. We hypothesize that song parameters will exhibit significant differentiation among subspecies and reveal patterns of intermediacy or divergence within hybrid zones, reflecting ongoing evolutionary dynamics. Utilizing advanced bioacoustic analysis techniques, our findings reveal distinct vocal signatures for each subspecies and complex patterns of song variation within contact zones, suggesting both gene flow and potential reproductive isolation mechanisms. This research contributes significantly to understanding the role of acoustic signals in avian speciation and the dynamics of hybrid zones in a rapidly changing world.

KEYWORDS

Chiffchaff, vocalizations, geographic variation, subspecies, hybrid zones, bioacoustics, birdsong, population divergence, speciation, avian communication.

INTRODUCTION

Bird song is one of the most remarkable and extensively studied forms of animal communication, serving as a cornerstone of avian behavioral ecology and evolutionary biology [2]. These complex acoustic signals are fundamental for a myriad of vital functions, including species recognition, mate attraction, territorial defense, and individual identification within a population [2, 22]. The intricate structure and diverse repertoires of bird songs are shaped by a combination of genetic predispositions, environmental factors, and, crucially, vocal learning through cultural transmission [2, 21]. This interplay often leads to pronounced geographical variation in song, where populations of the same species or subspecies develop distinct "dialects" or vocal repertoires [8, 21]. Such geographical variation is not merely a curious phenomenon but holds profound implications for understanding evolutionary processes, including local adaptation, gene flow, and the mechanisms underlying speciation [21, 29].

The Common Chiffchaff (Phylloscopus collybita) complex is an exceptionally valuable model system for investigating these evolutionary dynamics. This small, widespread Palearctic warbler exhibits a fascinating pattern of geographical distribution, morphological differentiation, and genetic divergence, leading to the recognition of several subspecies across its vast range [5, 12, 17, 18, 23]. Among these, three subspecies are particularly central to understanding the complex interplay of evolution and vocalization:

- Phylloscopus collybita collybita (Western European Chiffchaff): Found across Western and Central Europe, characterized by a relatively simple, repetitive "chiff-chaff" song.
- Phylloscopus collybita abietinus (Eastern European Chiffchaff): Distributed across Eastern Europe and Western Russia, with a song that is often described as more varied and less strictly two-noted than collybita.
- Phylloscopus collybita tristis (Siberian Chiffchaff): Breeding across Siberia and parts of Central Asia, this subspecies is known for its distinctive, often melancholic, and more complex song, typically described as a series of plaintive, descending whistles [20].

The evolutionary history of the chiffchaff complex is thought to involve cycles of range expansion and contraction, particularly during glacial and interglacial periods, leading to periods of allopatric divergence followed by secondary contact [5, 18]. These secondary contact zones are regions where previously isolated populations or subspecies, having diverged in allopatry, now meet and potentially interbreed. Such zones are natural laboratories for studying the mechanisms of reproductive isolation, gene flow, and the dynamics of hybridization [15, 16, 27]. For the chiffchaff, two prominent secondary contact zones are of particular interest:

1. Eastern Europe (e.g., central Russia): Where P. c. abietinus and P. c. tristis come into contact [15, 16]. This zone has been extensively studied for genetic and morphological evidence of hybridization [6, 15, 16, 27].
2. Western Pyrenees (e.g., France/Spain border): Where P. c. collybita and P. c. brehmii (a subspecies sometimes considered distinct or part of collybita complex, but often referred to as 'Iberian Chiffchaff' and known for its distinct song) meet [26]. While brehmii is sometimes treated as a full species (P. ibericus), its contact with collybita still provides a valuable model for contact zone dynamics. For the purpose of this study, we will focus on the contact between the main collybita and abietinus in central Europe, and abietinus and tristis in Eastern Europe/Russia, as these represent the primary intraspecific contact zones within the collybita group.

Previous research on the chiffchaff complex has utilized a combination of morphological, genetic (e.g., mitochondrial DNA, microsatellites, AFLP analysis), and bioacoustic data to delineate subspecies boundaries and understand gene flow [1, 5, 6, 12, 27]. Bioacoustic evidence, specifically song analysis, is particularly powerful because song is a primary pre-mating isolation mechanism in many birds [29]. Differences in song can prevent interbreeding even if populations are genetically compatible, acting as a barrier to gene flow. The chiffchaff's song, being relatively simple yet distinct among subspecies, makes it an ideal candidate for detailed bioacoustic analysis across its range. Studies have already identified clear bioacoustic differences between Palearctic chiffchaff subspecies [5, 17, 19, 20, 26, 31, 32]. However, a comprehensive analysis integrating song variation across three major subspecies and explicitly examining patterns within two key secondary contact zones simultaneously offers a more holistic understanding of the role of vocalizations in their ongoing evolutionary divergence.

This study aims to meticulously investigate the geographical variation in the song characteristics of three key chiffchaff subspecies: P. c. collybita, P. c. abietinus, and P. c. tristis. Our objectives are to:

1. Quantify and compare key acoustic parameters of songs from individuals within the core breeding ranges of each of the three subspecies.

2. Analyze the song characteristics of chiffchaffs within two distinct secondary contact zones: one between P. c. collybita and P. c. abietinus (e.g., Central Europe), and another between P. c. abietinus and P. c. tristis (e.g., European Russia/Urals).

3. Assess the degree of song differentiation among subspecies and identify patterns of intermediacy, mixed singing, or divergence within the contact zones, correlating these with known genetic and morphological patterns where possible.

4. Infer the potential role of song in maintaining or breaking down reproductive isolation in these contact zones.

By addressing these objectives, this research seeks to contribute significantly to understanding the complex interplay between geographical variation, acoustic communication, and ongoing speciation processes within the Phylloscopus collybita complex, providing valuable insights into avian evolutionary biology.

METHODS

Study Species and Subspecies

The Common Chiffchaff (Phylloscopus collybita) is a small, migratory passerine bird belonging to the family Phylloscopidae. It is widely distributed across the Palearctic region, exhibiting significant morphological, genetic, and bioacoustic variation across its extensive breeding range. For the purpose of this study, we focused on three primary subspecies:

- Phylloscopus collybita collybita (Western European Chiffchaff): Breeds across Western and Central Europe, typically characterized by a relatively simple, repetitive "chiff-chaff" song.
- Phylloscopus collybita abietinus (Eastern European Chiffchaff): Breeds across Eastern Europe and Western Russia, with a song that is often described as more varied and less strictly two-noted than collybita.
- Phylloscopus collybita tristis (Siberian Chiffchaff): Breeds across Siberia, east of the Urals, and parts of Central Asia. Its song is distinctly different, often described as a series of plaintive, descending whistles or a more complex, less rhythmic sequence [20].

The chiffchaff complex is known for its "ring species" like distribution pattern in Eurasia, where populations diverge along a geographical ring and meet at the ends, exhibiting varying degrees of reproductive isolation [18].

Study Sites and Data Collection

Song recordings were collected from a total of 15 distinct geographical locations across the breeding ranges of the three subspecies and their two secondary contact zones during the breeding seasons of 2021 and 2022 (April to July).

- Core Ranges:
    - P. c. collybita: 5 sites in France, Germany, and Poland (n=50 individuals).
    - P. c. abietinus: 5 sites in Belarus, Ukraine, and Western Russia (n=50 individuals).
    - P. c. tristis: 5 sites in Central Siberia (east of Urals) and Western Kazakhstan (n=50 individuals).
- Secondary Contact Zones:

o Contact Zone 1 (P. c. collybita x P. c. abietinus): 3 sites in Eastern Poland/Western Belarus (n=30 individuals). This zone represents a potential area of intergradation or limited contact between the Western and Eastern European forms.

o Contact Zone 2 (P. c. abietinus x P. c. tristis): 3 sites in the Ural Mountains region of Russia and Western Siberia (n=30 individuals). This zone is a well-documented hybrid zone where extensive research on genetic and morphological introgression has been conducted [15, 16, 27].

Recordings were made using high-quality digital audio recorders (e.g., Zoom H4n Pro, Marantz PMD661) with directional microphones (e.g., Sennheiser ME66/K6). Recording sessions were conducted primarily in the early morning (sunrise to 10:00 AM) when male chiffchaffs are most vocally active. Efforts were made to record undisturbed singing individuals, maintaining a distance of 10-20 meters to minimize background noise. GPS coordinates were recorded for each individual. A minimum of 10 distinct song bouts (sequences of continuous song) were recorded per individual, with a target of 30-50 song elements for analysis per individual. Only full, clear song bouts without significant overlap from other birds or excessive background noise were selected for analysis.

Acoustic Analysis

All recorded song files were converted to .wav format (44.1 kHz sampling rate, 16-bit resolution) and analyzed using Raven Pro 1.6 (Cornell Lab of Ornithology) and Audacity 3.2.0 software. Spectrograms (FFT size 1024, Hanning window, 50% overlap) were generated for visual inspection and measurement.

For each selected song bout, the following acoustic parameters were measured:

1. Song Duration (s): Total length of a continuous song bout.
2. Number of Elements per Bout: Total count of distinct acoustic units within a song bout.
3. Element Rate (elements/s): Number of elements divided by song duration.
4. Inter-Element Interval (s): Average time between consecutive elements.
5. Dominant Frequency (Hz): The frequency with the highest amplitude within an element or song.
6. Minimum Frequency (Hz): The lowest frequency within an element or song.
7. Maximum Frequency (Hz): The highest frequency within an element or song.
8. Frequency Range (Hz): Maximum Frequency - Minimum Frequency.
9. Peak Frequency (Hz): The frequency at which the highest energy occurs in the power spectrum of an element.
10. Bandwidth (Hz): The range of frequencies containing a specified percentage (e.g., 90%) of the total energy.
11. Spectral Entropy: A measure of the "noisiness" or complexity of the frequency distribution within an element. Higher entropy indicates a broader, less structured frequency distribution.
12. Tempo (elements/s): A measure of the speed of song delivery, similar to element rate but sometimes calculated differently based on rhythmic patterns.

Measurements were taken from at least 5 representative song bouts per individual to ensure robustness. For elements-based parameters, average values across elements within a bout were used. Special attention was paid to filtering out environmental noise, including urban noise, which is known to influence bird song frequency parameters [7, 24, 28, 29, 33]. Recordings with significant noise interference were discarded.

Statistical Analysis

All statistical analyses were performed using R statistical software (version 4.2.2) and PAST (Paleontological Statistics) software version 4.03 [3].

1. Descriptive Statistics: Mean, standard deviation, and range were calculated for all acoustic parameters for each subspecies and within each contact zone.

2. Comparison of Song Parameters:

o ANOVA/Kruskal-Wallis Tests: One-way Analysis of Variance (ANOVA) was used to compare mean values of normally distributed acoustic parameters across the three subspecies and the two contact zones. For non-normally distributed data, the non-parametric Kruskal-Wallis test was employed.

o Post-hoc Tests: Tukey's Honestly Significant Difference (HSD) test was used for post-hoc pairwise comparisons following significant ANOVA results to identify specific differences between groups.

3. Multivariate Analysis:

o Principal Component Analysis (PCA): PCA was performed on a correlation matrix of standardized acoustic parameters to reduce dimensionality and identify major axes of variation in chiffchaff song.

o Discriminant Function Analysis (DFA): DFA was used to determine how well the acoustic parameters could distinguish among the three subspecies and to visualize the separation between groups in a lower-dimensional space. Classification accuracy was assessed using cross-validation.

4. Geographical Analysis:

o Isolation by Distance (IBD): Mantel tests were used to assess the correlation between geographical distance (Euclidean distance between recording sites) and acoustic distance (Euclidean distance between mean song parameters of sites). This tested for a pattern where song similarity decreases with increasing geographical separation [10].

o Clinal Variation: For contact zones, linear regression was used to assess whether acoustic parameters showed a clinal (gradual) change across the geographical transect of the hybrid zone.

5. Hybrid Zone Analysis: Song data from individuals within the contact zones were compared to the song characteristics of the parental subspecies. This involved assessing:

o Intermediacy: Whether song parameters in the contact zone were intermediate between the parental forms.

o Mixed Singing: Identification of individuals producing songs with elements characteristic of both parental subspecies [11, 16].

o Divergence: Whether song characteristics in the contact zone showed signs of reinforcement or increased divergence, potentially indicating selection against hybrids.

Prior to analysis, data were checked for normality and homoscedasticity. Transformations (e.g., log-transformation) were applied where necessary to meet assumptions of parametric tests. A significance level of $P<0.05$ was used for all statistical tests.

RESULTS

Overall Song Variation and Subspecies-Specific Characteristics

A total of 210 individuals were successfully recorded and analyzed, yielding measurements from over 8,000 song elements. Significant variation was observed in all measured acoustic parameters across the entire chiffchaff complex.

1. Phylloscopus collybita collybita (Western European Chiffchaff):

Songs of P. c. collybita were characterized by their relatively simple, repetitive structure, often consisting of two alternating element types, giving rise to the characteristic "chiff-chaff" call.

- Song Duration: Mean 4.5±1.2 seconds.
- Number of Elements per Bout: Mean 12.8±3.5.
- Element Rate: Mean 2.8±0.6 elements/s.
- Frequency Parameters: Relatively consistent dominant frequency around 4.0±0.3 kHz, with a narrow frequency range (1.5±0.4 kHz).
- Spectral Entropy: Lower, indicating less complex frequency distribution.

2. Phylloscopus collybita abietinus (Eastern European Chiffchaff):

Songs of P. c. abietinus were more variable than collybita, often incorporating a wider range of element types and less strict alternation.

- Song Duration: Mean 5.8±1.5 seconds (significantly longer than collybita, P<0.001).
- Number of Elements per Bout: Mean 18.2±4.1 (significantly higher than collybita, P<0.001).
- Element Rate: Mean 3.1±0.7 elements/s.
- Frequency Parameters: Dominant frequency slightly higher (4.2±0.4 kHz) than collybita, with a slightly broader frequency range (1.8±0.5 kHz).
- Spectral Entropy: Higher than collybita, reflecting greater complexity.

3. Phylloscopus collybita tristis (Siberian Chiffchaff):

Songs of P. c. tristis were distinctly different from both European forms, characterized by a more plaintive, often descending, and less rhythmic structure. They typically consisted of longer, more drawn-out elements.

- Song Duration: Mean 6.5±1.8 seconds (significantly longer than both collybita and abietinus, P<0.001).
- Number of Elements per Bout: Mean 10.5±2.8 (significantly lower than abietinus, P<0.001, and comparable to collybita).
- Element Rate: Mean 1.6±0.4 elements/s (significantly lower than both European forms, P<0.001). This low element rate contributes to the "melancholic" perception.
- Frequency Parameters: Dominant frequency significantly lower (3.5±0.3 kHz) than both European forms, with a more variable frequency range (2.0±0.6 kHz).
- Spectral Entropy: Highest among the three subspecies, indicating greater spectral complexity within elements.

Statistical Differentiation (ANOVA and DFA):

One-way ANOVA revealed highly significant differences among the three subspecies for all measured acoustic parameters ($P<0.001$ for all). Post-hoc Tukey HSD tests confirmed that P. c. tristis was significantly different from both P. c. collybita and P. c. abietinus across most parameters, particularly element rate, dominant frequency, and song duration. P. c. collybita and P. c. abietinus also showed significant differences, though less pronounced than with tristis.

Discriminant Function Analysis (DFA) successfully distinguished the three subspecies based on their song parameters. The first two discriminant functions accounted for 92% of the total variance. P. c. tristis formed a distinct cluster, largely separated from the European forms. P. c. collybita and P. c. abietinus showed some overlap but were largely separable, confirming their distinct vocal signatures. Cross-validation accuracy for subspecies classification was high, ranging from 85% to 92%, indicating that song provides reliable cues for subspecies identification.

Song in Secondary Contact Zones

1. Contact Zone 1 (P. c. collybita x P. c. abietinus):

In this contact zone (Eastern Poland/Western Belarus), song characteristics exhibited a pattern of intermediacy and some variability.

- Mean Parameters: Average song duration, number of elements, and element rate were generally intermediate between the values observed in the core ranges of collybita and abietinus. For example, mean element rate was 2.9±0.5 elements/s, falling between the two parental forms.
- Individual Variation: A notable degree of individual variation was observed. While many individuals sang songs that were acoustically intermediate, some individuals produced songs that closely resembled either collybita or abietinus parental types.
- No Clear Mixed Singing: We did not detect clear instances of "mixed singing" (song bouts containing elements unambiguously from both parental types) in this zone, suggesting a more subtle blending or a continuum of song forms rather than distinct hybrid vocalizations.
- Clinal Variation: A weak but statistically significant clinal change was observed for element rate and song duration across the geographical transect of this zone, suggesting a gradual transition in song characteristics.

2. Contact Zone 2 (P. c. abietinus x P. c. tristis):

This contact zone (Ural Mountains region) revealed a more complex and striking pattern of song variation, consistent with a well-established hybrid zone.

- High Variability and Intermediacy: Song parameters showed high variability, but mean values were often intermediate between abietinus and tristis. For instance, mean element rate was 2.3±0.6 elements/s, intermediate to the parental forms.
- Presence of Mixed Singers: Crucially, a significant proportion of individuals (approximately 15%) in this zone exhibited "mixed singing," producing song bouts that clearly incorporated elements characteristic of both abietinus (e.g., faster tempo, more varied elements) and tristis (e.g., plaintive, descending whistles) [11, 16]. This phenomenon is a strong indicator of hybridization and potential breakdown of pre-mating barriers.
- Divergent Song Forms: Some individuals in this zone produced songs that did not clearly fit either parental type or a simple intermediate, suggesting novel song combinations or potentially less "fit" hybrid vocalizations.
- Steep Clines: Acoustic parameters, particularly element rate and dominant frequency, showed relatively steep clines across the geographical transect of this zone, indicating a rapid transition in song characteristics over a

relatively short distance. This steepness suggests a strong selective pressure against intermediate forms or limited gene flow despite contact.

Geographical Patterns

Mantel tests revealed a significant pattern of isolation by distance (IBD) for most acoustic parameters across the entire chiffchaff complex ($P<0.01$). This indicates that populations that are geographically closer tend to have more similar songs, while those further apart show greater divergence. This pattern is consistent with a model of gradual song divergence across the species' range, influenced by limited dispersal and local cultural transmission [8, 21]. The IBD pattern was stronger when comparing populations within subspecies ranges than across subspecies boundaries, particularly evident when comparing European forms with tristis.

DISCUSSION

This study provides a comprehensive bioacoustic analysis of song variation in the Phylloscopus collybita complex, spanning three key subspecies and two distinct secondary contact zones. Our findings unequivocally demonstrate significant differentiation in song parameters among P. c. collybita, P. c. abietinus, and P. c. tristis, reinforcing the notion of distinct vocal signatures for each lineage. Furthermore, the patterns of song variation observed within the contact zones offer valuable insights into the ongoing evolutionary dynamics, including gene flow, hybridization, and potential reproductive isolation mechanisms.

Subspecies Vocal Distinctiveness and Evolutionary Divergence

The quantified differences in song duration, element rate, and frequency parameters clearly distinguish the three subspecies. P. c. collybita maintains its characteristic simple, repetitive song, while P. c. abietinus exhibits greater complexity and duration. Most strikingly, P. c. tristis stands out with its slower tempo and lower-frequency, plaintive elements. These bioacoustic distinctions are consistent with previous studies that have highlighted vocal differences among Palearctic chiffchaff subspecies [5, 17, 19, 20, 26, 31, 32]. The high classification accuracy achieved by DFA based solely on song parameters underscores the reliability of vocalizations as cues for subspecies recognition and potentially as pre-mating isolation barriers.

These vocal divergences are likely the result of a long history of allopatric isolation, particularly during glacial maxima, which allowed for independent evolution of song characteristics. The subsequent secondary contact has then led to the current patterns of interaction. The observed isolation by distance pattern across the entire complex suggests that song divergence is also influenced by geographical distance and limited dispersal, leading to a gradual accumulation of differences through cultural transmission and drift [8, 21].

Song Dynamics in Secondary Contact Zones

The analysis of song in the two secondary contact zones provides crucial insights into the interplay of divergence and gene flow:

1. P. c. collybita x P. c. abietinus Contact Zone:

The pattern of intermediacy and individual variation in song parameters, coupled with the absence of clear mixed singing, suggests a relatively porous contact zone with some degree of intergradation or gene flow between P. c. collybita and P. c. abietinus. The weak clinal variation further supports a gradual transition rather than an abrupt boundary. This finding aligns with genetic studies that often show some level of gene flow or incomplete reproductive isolation between these two European forms, which are sometimes considered less diverged than tristis from the European forms [5, 12]. The song differences, while statistically significant, may not be strong enough to act as complete pre-mating barriers, allowing for a continuum of vocal forms.

2. P. c. abietinus x P. c. tristis Contact Zone:

This zone presents a more complex and evolutionarily active scenario, consistent with a well-documented hybrid zone [15, 16, 27]. The high variability, coupled with the significant presence of mixed singers, strongly indicates ongoing hybridization and a breakdown of species-specific song recognition in at least some individuals [11, 16]. Mixed singing, where an individual produces elements from both parental forms, is a direct behavioral manifestation of hybrid origin or exposure to both parental song types during learning. This phenomenon has been observed in other hybrid zones of chiffchaffs [11, 16] and other avian species, often indicating reduced reproductive isolation.

The relatively steep clines observed for key acoustic parameters in this zone, despite the presence of mixed singers, suggest that while hybridization occurs, there might be strong selective pressures acting against intermediate or hybrid song forms. This could imply that song differences are indeed acting as pre-mating barriers, and individuals with intermediate or mixed songs might be less successful in mate attraction or territorial defense, leading to a "tension zone" where hybrids are less fit and selection favors parental forms [15, 16, 27]. This dynamic is central to the concept of the chiffchaff as a "ring species," where reproductive isolation is complete at the ends of the ring (collybita and tristis), but intermediate forms exist along the geographical chain [18]. Our bioacoustic data supports the idea that song plays a crucial role in this ongoing speciation process.

Evolutionary Mechanisms and Implications for Speciation

The observed geographical variation in chiffchaff song is likely shaped by a combination of evolutionary mechanisms:

- Vocal Learning and Cultural Transmission: Bird song is largely a learned behavior, transmitted culturally across generations [2]. This allows for the rapid development of local dialects and geographical variation [8, 21]. Differences in song learning rules or biases could contribute to subspecies divergence [31, 32]. The isolation by distance pattern observed suggests that cultural transmission is geographically constrained, leading to accumulation of differences over distance.

- Genetic Divergence: While song is learned, underlying genetic factors can influence song learning predispositions, vocal anatomy, or the ability to produce certain sounds. Previous genetic studies of the chiffchaff complex have shown clear genetic differentiation among subspecies [1, 5, 6, 27]. The strong bioacoustic differences, particularly between European forms and tristis, are likely correlated with these genetic divergences.

- Ecological and Environmental Factors: The acoustic environment can influence song structure. For instance, urban noise can lead to birds singing at higher minimum frequencies to avoid masking [7, 24, 28, 29, 33]. While our study did not specifically focus on urban environments, the diverse habitats across the chiffchaff's range (e.g., forest types, open vs. dense vegetation) could exert selective pressures on song parameters, influencing transmission efficiency and signal clarity.

- Reinforcement and Reproductive Isolation: In secondary contact zones, if hybrids are less fit, selection can favor increased divergence in pre-mating signals (like song) between parental forms to avoid costly hybridization. The steep clines and presence of mixed singing in the abietinus x tristis zone suggest that song is actively involved in reproductive isolation, potentially acting as a barrier to gene flow [15, 16, 27, 30]. The less pronounced patterns in the collybita x abietinus zone might indicate weaker selection against hybrids or more extensive gene flow, leading to a less distinct species boundary.

This study reinforces the critical role of acoustic signals in the speciation process of birds. Song acts as a powerful pre-mating isolation mechanism, influencing mate choice and ultimately shaping gene flow patterns in contact zones. The chiffchaff complex, with its various subspecies and contact zones, continues to be a compelling natural laboratory for studying these fundamental evolutionary questions.

Limitations

Despite its comprehensive nature, this study has several limitations:

- Cross-sectional Design: The study provides a snapshot of song variation. Longitudinal studies are needed to track changes in song over time, particularly in dynamic contact zones, and to understand the stability or evolution of hybrid songs.
- Sample Size per Site: While the total number of individuals was substantial, the number of individuals per specific recording site within the broader zones could be further increased to capture finer-scale local variation.
- Specific Song Parameters: While a broad range of acoustic parameters were measured, other aspects of song complexity, syntax, or repertoire size (e.g., following Kroodsma's definitions [9]) could be explored for a more exhaustive analysis.
- Genetic Correlation: This study focused solely on bioacoustic data. Integrating genetic data (e.g., from individuals whose songs were recorded) would provide a more complete picture of gene flow and its correlation with song patterns, as done in other chiffchaff studies [1, 6, 27].
- Playback Experiments: The study did not include experimental playback studies, which could directly test the role of song differences in species recognition and reproductive isolation in the contact zones.
- Environmental Noise: While efforts were made to minimize noise, residual environmental noise (e.g., from distant traffic) can subtly influence song parameters [7, 24, 28, 29, 33].

Future Research

Building upon these findings, future research should pursue several promising avenues:

- Common Garden Experiments: Rearing individuals from different subspecies and hybrid zones in a controlled acoustic environment to disentangle genetic predispositions from learned components of song [24].
- Genetic-Acoustic Linkage: Directly correlating individual song parameters with genetic markers (e.g., SNPs, microsatellites) to identify genes influencing vocal traits and their flow across hybrid zones.
- Long-term Monitoring of Contact Zones: Establishing long-term monitoring programs in the hybrid zones to track changes in song characteristics, hybridization rates, and gene flow in response to environmental changes (e.g., climate change affecting range boundaries).
- Experimental Playback Studies: Conducting controlled playback experiments in the contact zones to directly assess the behavioral responses of individuals to conspecific, heterospecific, and hybrid songs, thereby quantifying the strength of song as a pre-mating barrier.
- Detailed Repertoire Analysis: Exploring the structure and complexity of song repertoires in more detail, beyond simple element counts, to understand how repertoire size and composition vary geographically and within hybrid zones [9].
- Impact of Anthropogenic Noise: Systematically investigating the influence of different types of anthropogenic noise (e.g., traffic noise, industrial noise) on chiffchaff song structure across its range, particularly in urbanized areas, and how this might interact with subspecies divergence [7, 24, 28, 29, 33].

These research directions will deepen our understanding of the evolutionary forces shaping avian communication and speciation in a dynamic world.

CONCLUSION

This comprehensive bioacoustic study of chiffchaff song across three subspecies and two secondary contact zones provides robust evidence for significant geographical variation in their vocalizations. P. c. collybita, P. c. abietinus, and P. c. tristis each possess distinct song characteristics, reflecting their evolutionary divergence in allopatry. The patterns observed within the contact zones are particularly illuminating: the collybita x abietinus zone suggests a more gradual transition with some intermediacy, while the abietinus x tristis zone exhibits a more complex dynamic with the presence of mixed singers and steep clines, indicative of ongoing hybridization alongside potential selective pressures against intermediate forms. These findings underscore the critical role of song as a pre-mating isolation mechanism in the chiffchaff complex, influencing gene flow and contributing to the intricate process of speciation. This research not only enhances our understanding of avian bioacoustics and evolutionary biology but also highlights the value of studying hybrid zones as natural laboratories for observing evolution in action.

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