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Incorporating Color into Integrative Taxonomy: Analysis of the Varied Tit (*Sittiparus varius*) Complex in East Asia

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Abstract.—Species designations are critically important scientific hypotheses that serve as the foundational units in a wide range of biological subdisciplines. A growing realization that some classes of data fail to delimit species under certain conditions has led to increasingly more integrative taxonomies, whereby species discovery and hypothesis testing are based on multiple kinds of data (e.g., morphological, molecular, behavioral, ecological, etc.). However, although most taxonomic descriptions have been based on morphology, some key morphological features, such as color, are rarely quantified and incorporated into integrative taxonomic studies. In this article, we applied a new method of ultraviolet digital photography to measure plumage variation in a color-variable avian species complex, the varied tit (*Sittiparus varius*). Plumage measurements corroborated species limits defined by morphometric, mitochondrial DNA, and nuclear DNA disjunctions and provided the only evidence for distinguishing two recently evolved species. Importantly, color quantification also provided a justification for lumping putative taxa with no evidence of evolutionary independence. Our revised taxonomy thus refines conservation units for listing and management and clarifies the primary units for evolutionary studies. Species tree analyses, which applied the newly delimited species as operational taxonomic units, revealed a robust phylogenetic hypothesis for the group that establishes a foundation for future biogeographic analyses. This study demonstrates how digital photography can be used to incorporate color character variation into integrative taxonomies, which should lead to more informed, more rigorous, and more accurate assessments of biodiversity. [Color, digital photography, integrative taxonomy, *Sittiparus varius*, species delimitation, varied tit.]

Species designations are critically important scientific hypotheses that provide a generalized framework for understanding historical as well as ongoing evolutionary interactions (Hey et al. 2003; Wheeler 2004). Working species hypotheses are initially generated by discovery approaches and are subsequently subject to continued testing and refinement through hypothesis testing approaches (Schlick-Steiner et al. 2010). Recently, there has been a strong push for "integrative taxonomy" (Will et al. 2005; Padial et al. 2010; Schlick-Steiner et al. 2010), whereby species discovery and hypothesis testing are based on multiple kinds of data (e.g., morphological, molecular, ecological, etc.). The need for integrative taxonomy springs from a growing realization that the geographic distribution of some types of data will fail to reflect species tree divergence under certain conditions. For example, mtDNA and other neutral genetic markers will fail to reveal recent divergence because of incomplete lineage sorting (Funk and Omland 2003; McKay and Zink 2010); likewise, morphological characters will fail to detect cryptic species that are divergent in other traits such as neutral genetic markers, ecological niches, pheromones, etc. (Bickford et al. 2007). The integration of multiple kinds of data exposes potential conflict and leads to more informed, more rigorous, and more accurate assessments of biodiversity (Padial et al. 2010).

A resurgent interest in species delimitation (Sites and Marshall 2003; Wiens 2007; Camargo and Sites 2013) has ushered in many new tools for species discovery and validation, but the majority of these methods are based exclusively on neutral DNA sequences (e.g., Pons et al. 2006; Ence and Carstens 2010; O'Meara 2010; Yang and Rannala 2010; Reid and Carstens 2013). A widely appreciated drawback to neutral molecular markers, which diverge through the weak force of drift, is that they might not contain adequate information about divergence if speciation is recent (Knowles and Carstens 2007; Zink and Barrowclough 2008; Fujita et al. 2012). This limits the capacity to falsify preexisting species hypotheses because the possibility that two putative species are real but recently evolved can never be ruled out. Indeed, a recent review by Carstens et al. (2013) found that only about 30% of recent species delimitation studies actually made any taxonomic recommendations. Further, all of the reported taxonomic recommendations involved splitting off new species, while none of the recommendations involved lumping preexisting species.

Ideally, species delimitation studies should be able to falsify prior species hypotheses, and this often

requires a critical reexamination of the characteristics that were originally described as differentiating taxa. However, although most taxonomic descriptions have been based on morphology, some key morphological features are rarely quantified. For example, color measurements are seldom incorporated into integrative taxonomic studies. This neglect may stem from several disadvantages associated with current color classification systems. Traditional color swatch matching methods (Ridgway 1912), while relatively inexpensive and easy to use, are prone to observer bias and cannot accommodate colors unseen by human vision (i.e., ultraviolet and infrared) (Endler 1990). In contrast, spectrophotometric methods (Anderson and Prager 2006), while objective and accurate, are generally expensive, time-consuming, and only provide small point measurements, which limit their usefulness for systematic applications. Advances in digital camera technology have recently made digital photography an attractive alternative to spectrophotometry (Stevens et al. 2007) with many advantages especially relevant for systematic applications (McKay 2013). In this article, we applied a new method of ultraviolet (UV) digital photography to incorporate color measurements, along with morphometrics, mitochondrial DNA (mtDNA) sequences, and nuclear DNA (nuDNA) intron sequences, into an integrative taxonomy of a morphologically variable species complex, aptly named the varied tit (*Sittiparus varius*). Our goal was to identify independent evolutionary lineages that could serve as the primary units in future evolutionary studies and conservation planning.

The varied tit is a small non-migratory passerine bird of broadleaf forest. Endemic to East Asia, it is largely confined to the major islands in the region including mainland Japan, the Ryukyu Islands, and Taiwan (Fig. 1). The complex consists of eight widely recognized subspecies (Dickinson 2003; Gosler and Clement 2007), and a ninth subspecies, *S. v. yakushimensis*, is also sometimes recognized (Ornithological Society of Japan 2012). Subspecies were described based on differences in size and coloration (Appendix). Two subspecies, *S. v. castaneoventris* and *S. v. owstoni*, were originally described as full species. Species-level taxonomy of the complex is of conservation concern because six subspecies (*S. v. sunsunpi*, *S. v. yakushimensis*, *S. v. namiyei*, *S. v. owstoni*, *S. v. olivaceus*, and *S. v. castaneoventris*) have small populations confined to one or a few small islands whereas another subspecies, *S. v. orii*, is presumed extinct. To the best of our knowledge, a comprehensive taxonomic evaluation of the complex has never been published.

Methods

Morphological Data Collection

Referring to the original taxonomic descriptions, we compiled a list of all morphological differences reported as distinguishing the nine subspecies (Appendix). Variation among these characters, which included morphometric measurements as well as plumage coloration, was quantified from 84 museum-vouchered specimens collected from across the range of all nine subspecies of the varied tit complex (Fig. 1; see Supplementary Table S1 for a complete list of voucher specimens measured; Dryad doi: http://dx.doi.org/10.5061/dryad.6q44t). To control for possible sexual dimorphism, only adult male specimens were examined. All measurements were made by the same person (B.D.M.).

Morphometric measurements were made following the methods described in Baldwin et al. (1931) and included bill length, bill depth, bill width, tarsus length, tail length, and wing chord. All bill measurements were made from the anterior edge of nare. Two composite variables, overall body size and bill size, were also mentioned in the original taxonomic descriptions. To represent overall body size, we used principal component (PC) I from a correlation matrix-based PC analysis of all morphometric log-transformed measurements following the recommendation of Rising and Somers (1989). Similarly, bill size was represented as PCI of a PC analysis of the three log-transformed bill measurements.

Plumage color differences were quantified using digital photography (Stevens et al. 2007). Ventral, dorsal, and lateral images of each specimen were taken using the equipment and following the protocol described by McKay (2013). This method uses a combination of human-visible and ultraviolet (UV) images to extract raw reflectance data at four points along the light spectrum: long wavelength (LW), medium wavelength (MW), short wavelength (SW), and ultraviolet wavelength (UV). Human-visible and UV images were independently calibrated to known reflectance standards and loaded as stacks into the program ImageJ (Schneider et al. 2012), so that the same plumage area could be selected simultaneously in both images. Selections were made of the largest area possible for each of nine plumage patches mentioned in the original taxonomic descriptions: throat band, nape streak, flanks, throat, back, frontal band, breast, black of head, and mantle (Appendix). Care was taken to avoid selecting areas with ruffled feathers or otherwise damaged plumage. The average raw LW, MW, SW, and UV reflectance measurements of each selection were converted into the opponent colorspace described by McKay (2013). This colorspace, which is based on one described by Endler (2012), reduces the four raw reflectance measurements into three more intuitive color coordinates: a red–blue opponent hue coordinate that varies from 1 (red) to –1 (blue), a green–ultraviolet hue opponent coordinate that varies from 1 (green) to –1 (ultraviolet), and a luminance (sometimes referred to as brightness) coordinate that is expressed as a percentage. As reported by Stoddard and Prum (2008), we found that very dark plumage patches yielded random hues, so hue measurements were excluded for plumage patches with <0.05 average luminance reflectance.

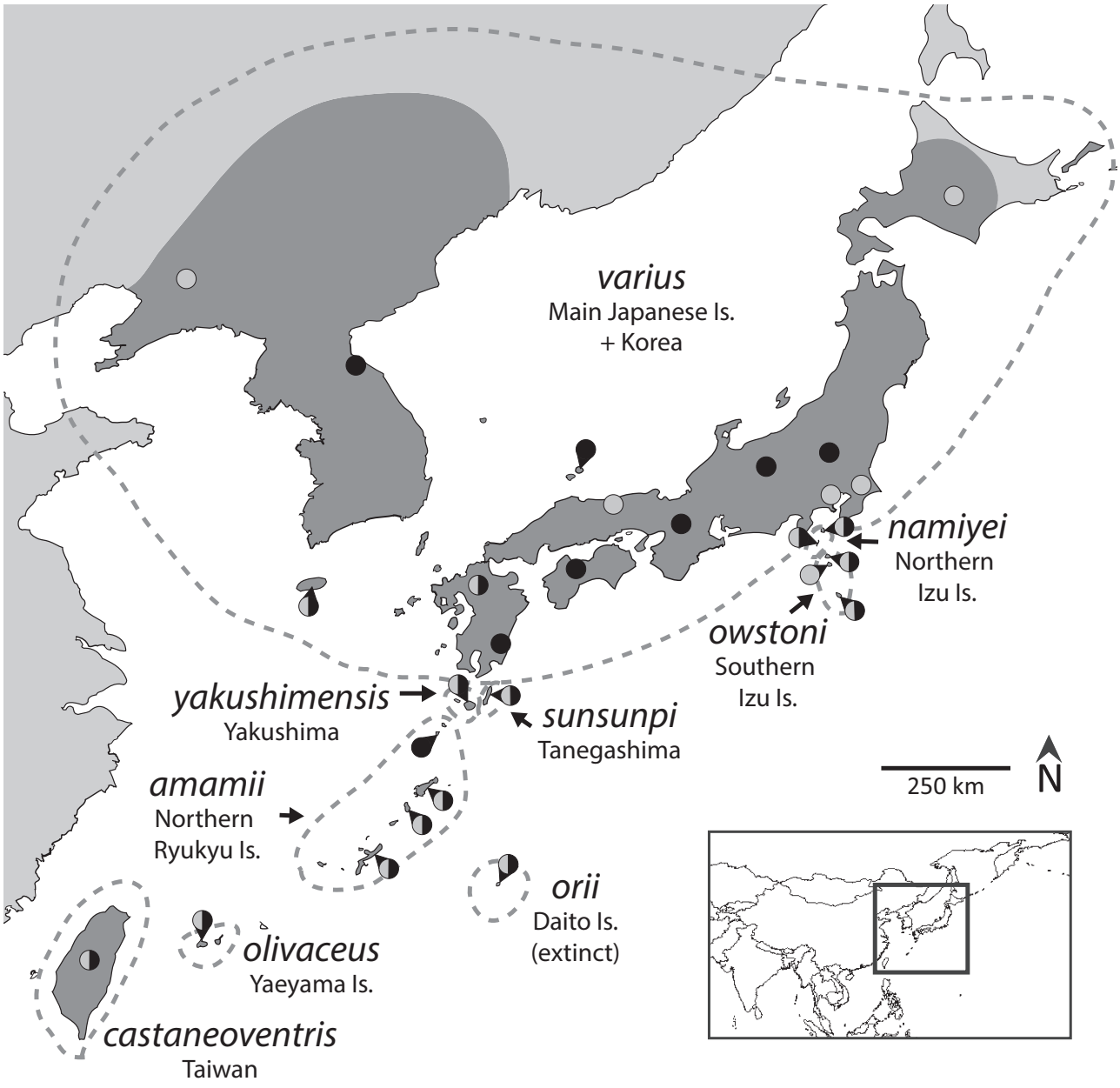

FIGURE 1. Map of East Asia showing the distributions (dark shaded areas outlined by dashed lines) of nine described taxa comprising the varied tit (*S. varius*) complex. Sampling sites are indicated by circles; black represents morphological samples and gray represents molecular samples.

Because specimen age is known to affect plumage color (Armenta et al. 2008), we tested for significant correlations among specimen age and opponent color space values using Pearson's correlation coefficients. We also evaluated any possible effects of seasonal plumage wear by testing for significant correlations among opponent color space values and ordinal dates of specimen collection, again using Pearson's correlation coefficients. All calibrated images have been deposited in Morphbank (Image 834477-834980; Collection 834986).

mtDNA Data Collection

We searched for morphologically similar but evolutionarily divergent cryptic species (Bickford et al. 2007) using an mtDNA survey. A total of 83 tissue samples were collected from across the range of all nine subspecies of the varied tit complex (Fig. 1; see Supplementary Table S2 for a detailed list of tissue specimens). Whole genomic DNA was extracted using a standard phenol-chloroform protocol followed by ethanol precipitation. The mitochondrial NADH

dehydrogenase subunit 2 (ND2) gene was amplified using the primers L5216 and H6313 (Sorenson et al. 1999). Polymerase chain reaction (PCR) and sequencing were performed following McKay (2013). Because no fresh tissue was available for the extinct taxon *S. v. orii*, mtDNA was extracted and sequenced from a small piece of toepad cut from a museum skin (AMNH681361). Toepad DNA was sequenced in nine overlapping fragments; DNA extraction, PCR, and sequencing protocols as well as a list of primers for toepad sequencing can be found in the Supplementary Table S3. Sequences were aligned and edited using Geneious v. 5 (Drummond et al. 2010). Phylogenetic relationships among mtDNA haplotypes using a maximum-likelihood approach with 100 replicates of non-parametric bootstrapping in the program PHYML (Guindon et al. 2010). Likelihood analysis was performed using the best-fit model of nucleotide substitution determined with Akaike information criteria (AIC) in the program jModeltest 2 (Darriba et al. 2012; Guindon and Gascuel 2003). All mtDNA sequences have been deposited in GenBank (KF932840-KF932924).

Integrative Taxonomy

We first attempted to validate previous taxonomic hypothesis using the (putative) character differences that were reported in the original taxonomic descriptions. Assessing character distributions between two putative taxa can result in at least three possible outcomes. First, character distributions may be non-overlapping (i.e., discrete). Second, distributions may overlap, but differ statistically. Third, there may be no evidence that character distributions are different. We characterized plumage overlap following McKay (2013). Briefly, plumage patch measurements were plotted in three-dimensional opponent colorspace using MATLAB, and minimum convex polygons were constructed around each subspecies using the "CONVHULLN" command. The presence of overlap among subspecies was then assessed by eye. Overlap among morphometric measurements was determined using boxplots. We tested for mean differences between subspecies pairs using *t*-tests. Prior to statistical analysis, morphometric data were log-transformed and plumage opponent coordinates, which are proportions, were arcsine transformed. For each plumage patch, *t*-tests were applied to each of the three color opponent coordinates separately, and a significant result for any one coordinate was considered a significant result for the plumage patch. Statistical analyses were performed SPSS 20 (IBM, Armonk, NY). We used an alpha level of 0.05 with Bonferroni correction applied to accommodate multiple comparisons.

We then integrated morphometric, plumage, and mtDNA data to generate a revised hypothesis of species limits within the varied tit complex. We defined species as independent evolutionary lineages (Wiley 1978; de Queiroz 1998), and used non-overlapping (i.e., fixed or diagnostic *sensu* Cracraft 1983) patterns of character distributions as operational criteria for recognizing species. We applied the criteria of non-overlapping trait differences rather than trait frequency differences because fixed differences are generally thought to indicate an absence of gene flow (i.e., evolutionary independence) in a way that frequency differences do not (Wiens 1999).

We identified non-overlapping morphometric and plumage character distributions as defined above. However, rather than limiting comparisons to those reported in the original taxonomic descriptions, we expanded the analysis to include comparisons of all morphological characters among all nine putative species. This made it possible to identify all morphological characters that were diagnosable in the context of the complex as a whole. We assessed fixed mtDNA base pair differences among subspecies by eye using ND2 sequence alignments. Finally, we integrated morphometric, plumage, and mtDNA to determine those subspecies identifiable by at least one non-overlapping character. These were treated as species in subsequent phylogenetic and biogeographic analyses.

Phylogenetic Analysis

Species delimited above were used as operational taxonomic units (OTUs) in a phylogenetic time-tree analysis. Within each species, we chose a geographically diverse subset of four individuals (eight alleles) for multi-locus sequencing. Following mtDNA protocols, we sequenced six nuclear introns (08352, 09385, 10179, 11887, 12884, and 15349) from primers described by Backström et al. (2008). Nuclear intron sequences were then combined with the mtDNA sequences to produce our multilocus data set. Although the white-fronted tit (*Cyanistes semilarvatus*) has recently been implicated as the sister taxon of the varied tit (*S. varius*) complex (Johansson et al. 2013), we were unable to obtain fresh tissue samples of white-fronted tit. Instead, individuals of white-browed tit (*S. superciliosus*) and Carolina chickadee (*S. carolinensis*), both close relatives of *S. varius* (Gill et al. 2005; Johansson et al. 2013), were sequenced for all loci and used as outgroups. All nuclear sequences have been deposited in GenBank (KF932733–KF932839).

The phase of nuclear alleles was determined computationally using PHASE 2.1 algorithms (Stephens et al. 2001) implemented in DnaSP v. 5 (Librado and Rozas 2009). Phased sequence alignments have been deposited in Dryad (doi: http://dx.doi.org/10.5061/dryad.6q44t). We looked for evidence of recombination using the Φ_w-statistic (Bruen et al. 2006) implemented in the program SplitsTree v. 4.10 (Huson and Bryant 2006), and tested for selection using the Hudson–Kreitman–Aguade (HKA) test (Hudson et al. 1987) implemented in DnaSP. We selected the best-fit model of nucleotide substitution for each locus using AIC implemented jModeltest 2. We used PAUP* v. 4.0b10 (Swofford 2003) to generate maximum-likelihood gene trees and tested

the molecular clock hypothesis for each locus using a likelihood ratio test. Statistical parsimony networks of nuclear alleles were constructed using the program TCS v. 1.21 (Clement et al. 2000).

Our phylogenetic time-tree analysis was based on a species tree approach implemented in the program *BEAST v. 1.7 (Heled and Drummond 2010; Drummond et al. 2012). *BEAST uses a Bayesian Markov chain Monte Carlo (MCMC) method to jointly estimate multiple gene trees embedded in a shared species tree. Because the molecular clock hypothesis was not rejected for any locus, we used strict molecular clock models for all loci. Gene trees were estimated using UPGMA starting topologies. For species tree priors, we used a Yule tree prior and a piecewise linear and constant root population size model. Nucleotide substitution model priors were set to default prior distributions. We estimated divergence dates using a molecular clock approach. To incorporate uncertainty in the mtDNA molecular clock, we used a range of ND2 substitution rates, from approximately 2.1% divergence per myr (0.0105 substitutions per site per myr (Weir and Schluter 2008)) to 5.52% divergence per myr (0.0276 substitutions per site per myr (Drovetski et al. 2004)). The substitution rates of nuclear loci were estimated in BEAST relative to the defined ND2 rate. We ran MCMCs for 100 million generations (sampling every 10,000 generations and discarding the first 10% as burn-in). Three independent MCMCs were run and their convergence was assessed in the program Tracer v. 1.5 (Rambaut and Drummond 2007). We conducted *BEAST analyses using all seven loci as well as using only the six nuclear loci (i.e., excluding mtDNA).

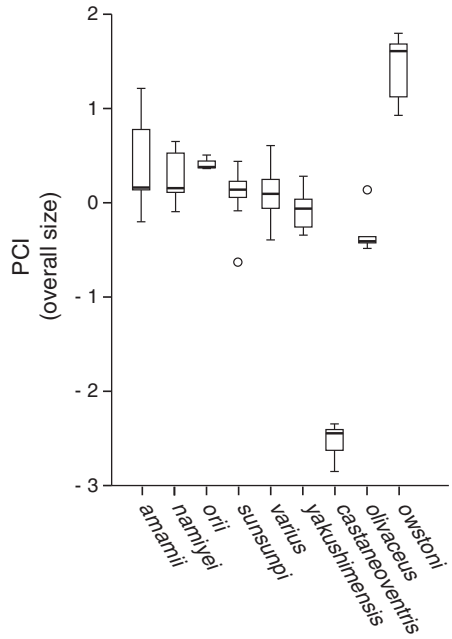

FIGURE 2. Boxplot showing overall size variation among subspecies of the varied tit (*S. varius*) complex. Overall size is represented by principle component (PC) I from a PC analysis of six morphometric measurements (bill length, bill depth, bill width, tarsus length, tail length, and wing chord). Heavy lines inside boxes represent the mediums (second quartiles). The taxon *castaneoventris* showed non-overlapping size variation from the other taxa. Although *owstoni* was significantly larger than the other taxa, its size distribution did overlap with some *amamii* individuals. Each box is bounded by the first and third quartiles, and plot whiskers extend to 1.5 times the interquartile range or to the maximum and minimum value, as appropriate. Data points outside the whisker range are represented by open circles.

RESULTS

Morphological and mtDNA Data

Morphometric measurements were variable both within and among subspecies of the varied tit. Size trends among morphometric characters were relatively constant, with the taxon *owstoni* largest and the taxon *castaneoventris* smallest, on average, in all measurements. Variation in overall body size typified the size trends found among subspecies and is shown with boxplots in Figure 2. Boxplots of other morphometric characters can be found in Supplementary Figure S4.

Plumage color measurements were also variable. The nine measured plumage patches ranged from highly discriminatory of subspecies (e.g., throat band) to almost completely homogeneous across subspecies (e.g., black of head). Three patches (mantle, throat, and black of head) had average luminance values <0.05, so their hue measurements were excluded. Luminance measurements from these three patches did not discriminate among subspecies (data can be found in Supplementary Fig. S5). Five of the six plumage patches for which full hue and luminance data were collected discriminated at least one subspecies. Three-dimensional colorspace plots of these patches are shown in Figure 3.

A total of 21 color variables (12 hue and 9 luminance) were quantified. After Bonferroni correction, four of these variables (i.e., breast green–ultraviolet, frontal band green–ultraviolet, back green–ultraviolet, and nape streak green–ultraviolet) were significantly correlated with specimen age. In each of these cases, there was a slight tendency for the ultraviolet contribution of the green–ultraviolet hue coordinate to decline with increasing specimen age. Although some colors are known to degrade over time, the plumage patches measured in this study were mostly browns and grays, colors that are reportedly less prone to fading (Armenta et al. 2008). However, dust may disproportionally absorb UV wavelengths (Griggio et al. 2011), so it is possible that gradually accumulating dust, rather than color degradation, is responsible for our observed age effects. Regardless of the cause, however, specimen age effects did not seem to alter any of the conclusions of this study. Further, no color variables were significantly correlated with the ordinal date of specimen collection, indicating that seasonal plumage wear did not affect our results.

We obtained all 1041 base pairs (bp) of the mtDNA ND2 gene. The ingroup alignment consisted

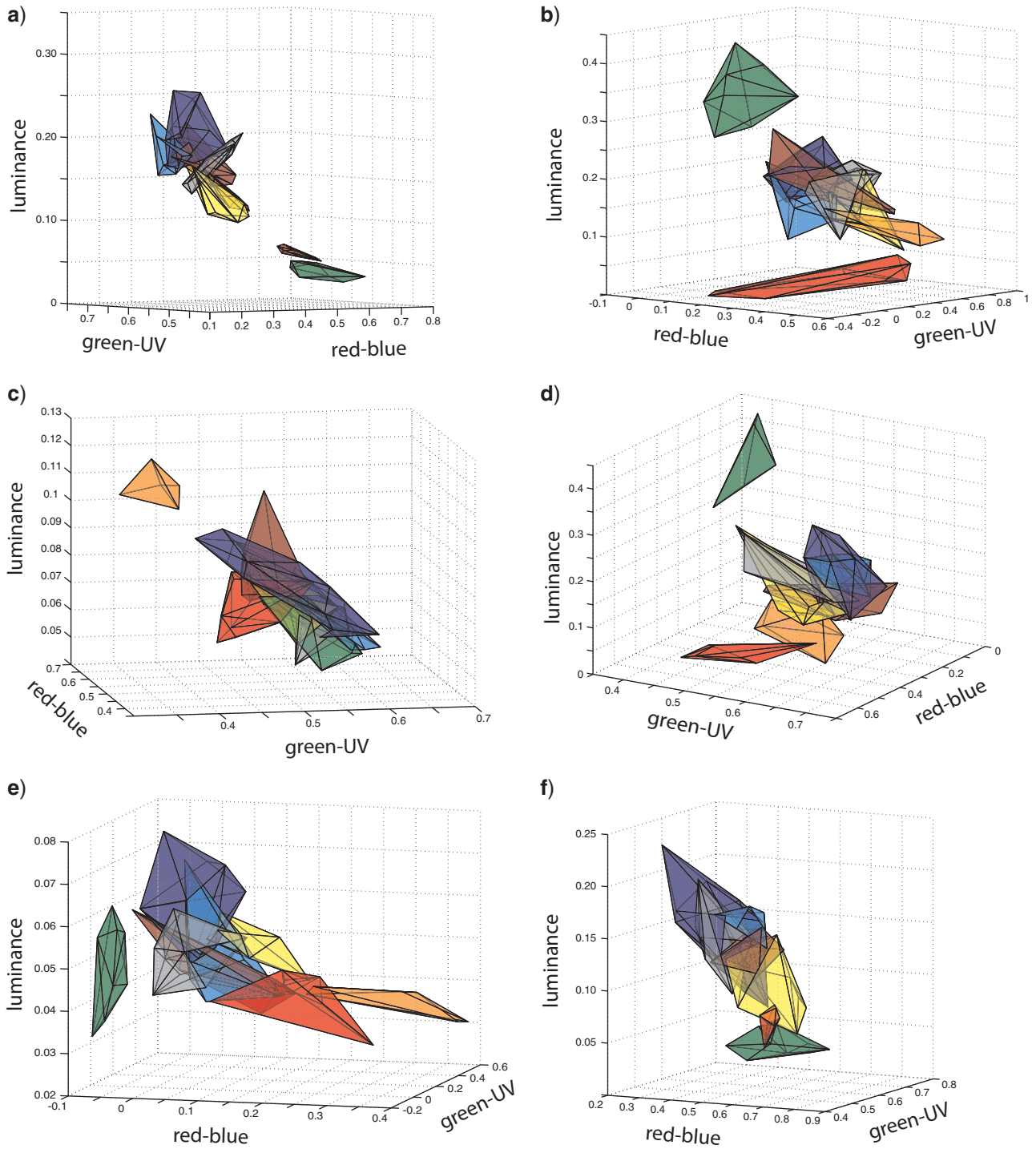

FIGURE 3. Three-dimensional plots of plumage measurements in opponent colorspace: a) throat band, b) nape streak, c) flanks, d) frontal band, e) back, and f) breast. To better illustrate character overlap, minimum convex polygons were constructed around the individual measurements of each subspecies and colored: *castaneoventris*, green; *olivaceus*, orange; *owstoni*, red; *amamii*, brown; *namiyei*, yellow; *orii*, pink; *sunsunpi*, light blue; *varius*, dark blue; *yakushimensis*, gray. Plots have been rotated independently to show non-overlapping components. The exception is the frontal band plot; *owstoni* and *olivaceus* are non-overlapping from each other and from all other taxa, but this is very slight and not apparent from the angle of the plot.

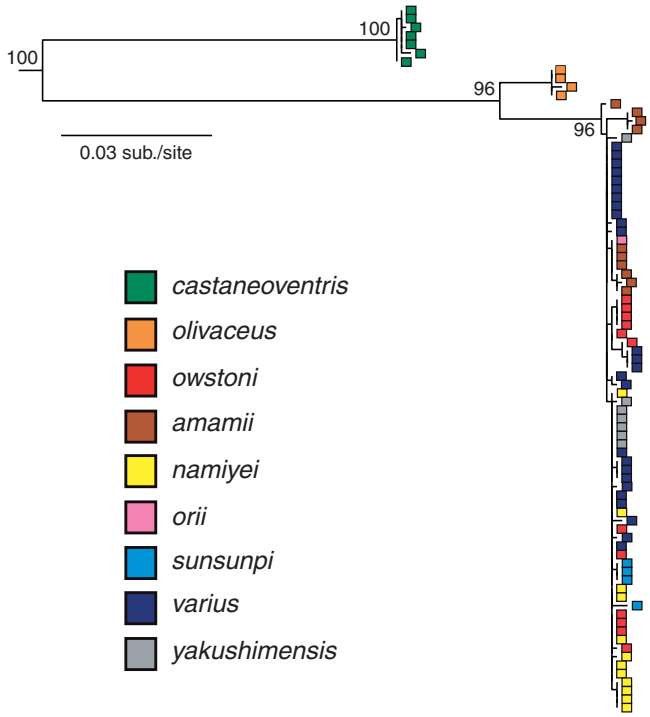

FIGURE 4. Maximum-likelihood phylogenetic analysis of ND2 sequences from the varied tit (*S. varius*) complex. The taxa *castaneoventris* and *olivaceus* formed well-supported reciprocally monophyletic groups, whereas the other seven taxa were all recovered in a single well-supported clade. Branch lengths are proportional to sequence divergence. Only bootstrap values >95 are shown. The tree was rooted with outgroups.

of 135 polymorphisms and 33 haplotypes. Phylogenetic analysis resolved three strongly supported reciprocally monophyletic groups: a group consisting of all individuals of the taxon *castaneoventris*, a group consisting of all individuals of the taxon *olivaceus*, and a group consisting of all individuals of the other seven taxa (Fig. 4).

Integrative Taxonomy

Referring to original taxonomic descriptions, we compiled a total of 53 specific statements describing morphological differences among the nine putative taxa; 19 statements involved morphometric differences, and 34 statements involved plumage differences (Appendix). The number of differences per subspecies varied from 1 (*castaneoventris*) to 10 (*namiyei*), and the average number of specified differences in each taxonomic description was 6.6. In each case, statements contrasted differences between the newly described taxon and a previously described taxon. All characters mentioned in the descriptions were measured with the exception of the amount of chestnut in the nape of the neck. Although color proportions are generally easy to quantify with digital photography (McKay 2008; McKay 2013), this character was difficult to accurately measure because the amount of exposed chestnut varied depending on the head angle of the specimen preparation. Three subspecies (*castaneoventris*, *olivaceus*, and *amamii*) were reported to differ in the amount of chestnut in their nape. Although this character is qualitatively variable, it was not included in our analyses.

When quantified, reported (putative) differences received varying levels of support. Twenty-five (47.2%) of the described differences were statistically significant, and 22 (41.5%) were non-overlapping. However, most of these non-overlapping differences did not hold up as diagnostic when considered in the context of the complex as a whole. For example, Kuroda (1923) described the nape streak of *S. v. orii* as being paler than *S. v. owstoni*, and, although the nape streak color distributions of *orii* and *owstoni* did not overlap with each other, the color distribution of *orii* did overlap with the color distribution of the geographically proximate *amamii*, which in turn broadly overlapped with the main *varius* color distribution. Therefore, in this case, although the taxonomic description did describe a non-overlapping difference, the character could not be considered as diagnostic for the taxon. Only five (9.4%) of the described differences were diagnostic when considered in the context of the complex as a whole; these characters identified three subspecies (*castaneoventris*, *olivaceus*, and *owstoni*) as taxa diagnosably distinct from a broadly defined *varius* taxon.

Expanding the analysis above to include all possible comparisons of every morphological character with every subspecies identified the same four species, but revealed additional diagnostic features (Fig. 5). Therefore, we recognized the following species within the varied tit complex: *S. varius*, *S. castaneoventris*, *S. olivaceus*, and *S. owstoni* (Fig. 6a). *S. castaneoventris* was characterized by the most fixed differences and was diagnostic for morphometric, plumage, mtDNA sequence, and nuDNA sequence characters. Likewise, *S. olivaceus* was characterized by a range of diagnostic characters including plumage, mtDNA sequence, and nuDNA sequence. *S. owstoni* was diagnostic for three plumage characters. We found no evidence of diagnostic differences within the putative taxa *amamii*, *namiyei*, *orii*, *sunsunpi*, *yakushimensis*, so we synonymized these forms with the taxon *S. varius*.

Sequences and Phylogenetic Analysis

We collected 3039 bp of nuclear intron sequence and 1041 bp of the mtDNA (ND2) sequence (4080 bp combined nuclear and mtDNA sequence per individual sample). Three loci had indels (of between 1 and 4 bp) within the ingroup that were identified by eye and removed prior to analyses. There were no missing data within the ingroup; outgroup sequences consisted of 13% missing data. No locus exhibited evidence of recombination or selection, and the hypothesis of a molecular clock could not be rejected for any locus. Two species, *S. castaneoventris* and *S. olivaceus*, had fixed nuclear differences, whereas *S. varius* and *S. owstoni*

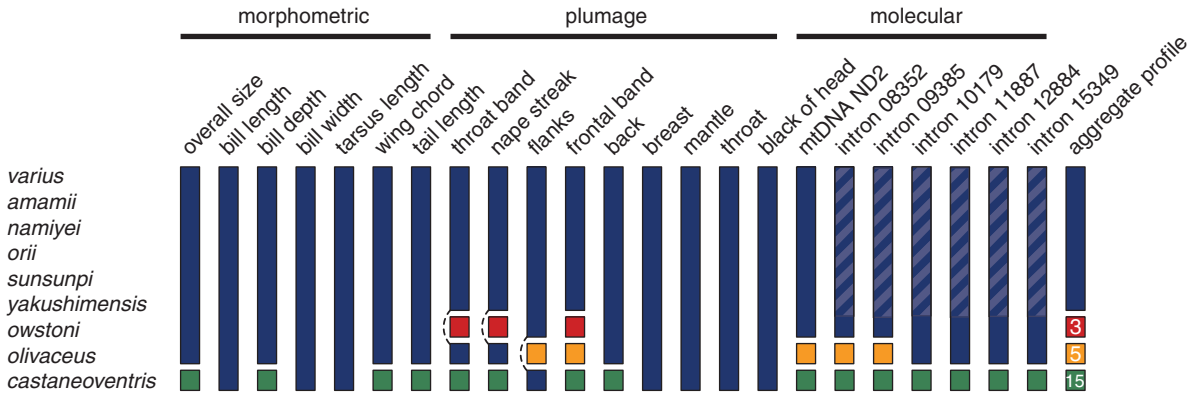

FIGURE 5. Cartoon summarizing all diagnostic (i.e., fixed or non-overlapping) characters for nine putative taxa of the varied tit (*S. varius*) complex. Characters are divided by type (i.e., morphometric, plumage, or molecular), and an aggregate profile summarizing all diagnostic characters is shown. Nuclear intron characters, which were not used to generate species hypotheses, are also shown. Note that nuclear intron data were limited or missing for several taxa, so nuclear intron character states for these taxa (indicated in stripes) were inferred from the integrative taxonomic hypothesis of four species: *S. varius*, *S. owstoni*, *S. olivaceus*, and *S. castaneoventris*.

shared alleles at every locus. Detailed information for individual loci is available in Supplementary Table S6, and nuclear allele networks for each locus can be found in Supplementary Figure S7.

The species tree analysis resulted in a strongly supported topology (Fig. 6b) that was consistent with the estimated mtDNA gene tree topology (Fig. 4). A basal split was recovered between *castaneoventris* and an *olivaceus/varius/owstoni* clade. The next split was between *olivaceus* and a *varius/owstoni* clade. The species *varius* and *owstoni* were recovered as sister taxa. The same topology was produced whether or not the mitochondrial ND2 gene was included in the analysis. Divergence time dating, which incorporated uncertainty in the ND2 substitution rate, indicated that the basal split between the *castaneoventris* and *olivaceus/varius/owstoni* clades occurred approximately 3.10 (1.62–4.68 highest posterior density (HSD)) million years (Ma) ago. The next split between the *olivaceus* and *varius/owstoni* clades occurred approximately 0.65 (0.29–1.02 HSD) Ma ago. The split between *varius* and *owstoni* occurred approximately 0.07 (0.003–0.13 HSD) Ma ago.

DISCUSSION

Integrative Taxonomy

Like many modern systematists (Wiens 2007), we defined taxa as evolutionary lineages, and treated species as metapopulation lineages that exist at the boundary between phylogeny and genealogy (i.e., tokogeny) (Wiley 1978; Mayden 1997; de Queiroz 1998). In this system, species are hypotheses about order in nature, and, as such, provide explanatory power as well as a predictive framework for understanding the distributions of characters among organisms. We made a distinction between this theoretical concept of species and the evidence used to recognize species (Frost and Kluge 1994; Mayden 1997). As operational criteria for species recognition, we employed a criterion of fixed (or diagnosable) character differences (Cracraft 1983; Nixon and Wheeler 1990). This evidence-based approach provides an empirical means of determining, in any given case, whether we are looking above (phylogeny) or below (tokogeny) the species boundary.

However, we recognize that earlier systematists did not necessarily operate under this framework or this view of taxa. For example, many of the taxa analysed in this species delimitation study were originally described as subspecies. The subspecies concept has long been controversial (Wilson and Brown 1953; Wiens 1982; Zink 2004). Some view subspecies as convenience and their description as an "art" (e.g., Fitzpatrick 2010), whereas others view subspecies more rigorously, synonymizing them with phylogenetic species (e.g., Remsen 2010). Historically, subspecies occupying all points between these extreme views have been described. Therefore, in this study, we critically tested taxa for properties they were not necessarily described as possessing. However, as others have noted (e.g., Zink 2004) and as our study demonstrates, the subspecies rank is currently a mix of independent evolutionary lineages as well as arbitrary divisions of geographically continuous variation. For example, the taxon *olivaceus* was originally described as a subspecies and has, to our knowledge, never been considered a species-level taxon. However, *olivaceus* fulfilled species criteria, possessing a range of diverse diagnostic features including plumage differences, mtDNA sequence differences, and multiple nuclear sequence differences. Thus, we suggest that subspecies are still potentially valuable as putative species hypotheses, and they provide a useful starting place for modern species delimitation studies.

The primary goal of this study was to refine taxonomic hypotheses within the varied tit, a morphologically

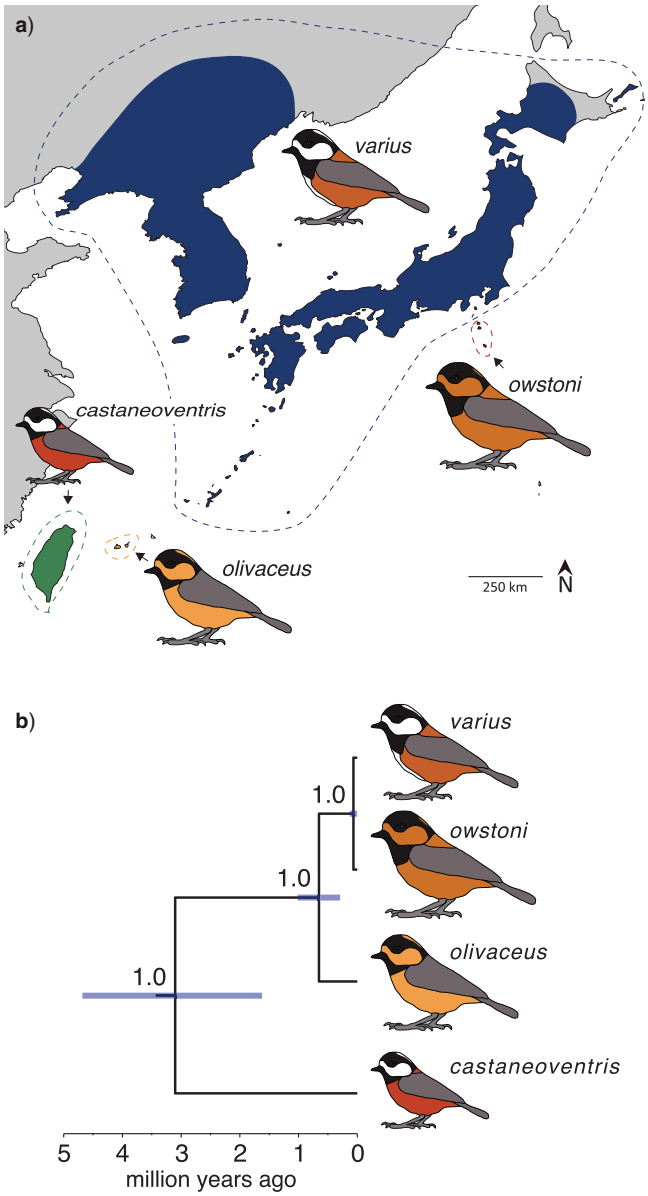

FIGURE 6. a) Map of East Asia showing the distributions (colored areas outlined by dashed lines) of the four taxa (*S. varius*, *S. castaneoventris*, *S. owstoni*, and *S. olivaceus*) recognized in this study. b) Species tree inferred from *BEAST showing the phylogenetic relationships among the four species. Purple bars represent HPDs of divergence time estimates inferred in *BEAST using mtDNA substitution rate estimates. Posterior probabilities of clade support are shown at each node. The tree was rooted with an outgroup. Cartoons are sized to scale based on average overall body size measurements.

variable species complex. High plumage variability, in particular, motivated a need to incorporate color measurements into the taxonomic revision. Plumage measurements were vital as they provided the only evidence for recognizing *S. owstoni* as a discrete evolutionary lineage. Because the original taxonomic descriptions were largely based on plumage differences, rigorous tests of color distributions also provided a justification for synonymizing five putative taxa for which there was no evidence of diagnostic differences. For example, *S. v. namiyei*, which occurs in the Northern Izu Islands, is geographically intermediate between *S. v. owstoni* in the Southern Izu Islands and *S. v. varius* in Mainland Japan and was described by Kuroda (1923) as also intermediate in phenotype (see also Yamaguchi 2005). The perception that *S. v. namiyei* bridged the phenotypic gap between *S. v. varius* and *S. v. owstoni* is perhaps a large part of the reason *S. v. owstoni* has not be re-elevated to species status. Our color measurements demonstrated that, although the color variation of several plumage patches in *S. v. namiyei* did generally fall between *S. v. varius* and *S. v. owstoni*, the range of variation found in *S. v. namiyei* overlapped broadly with *S. v. varius*, whereas there was a clear disjunction between *S. v. owstoni* and *S. v. namiyei*/*S. v. varius*. Color measurements thus showed that *S. v. owstoni* differed discretely in a way that *S. v. namiyei* did not. This situation would have been difficult to resolve without color quantification methods.

We found evidence for four species within the traditionally defined varied tit complex, and we propose the following English names: varied tit (*S. varius*), chestnut-bellied tit (*S. castaneoventris*), Owston's tit (*S. owstoni*), and Iriomote tit (*S. olivaceus*). We synonymized five previously described subspecies with *S. varius*, which we note is still an uncommonly variable taxon. For example, qualitative traits such as the presence of a contrasting breast streak or throat stripe as well as characteristics such as the extent of chestnut coloration on the back all differ to varying extents within and among populations of *S. varius*. We therefore recommended retaining the English name varied tit for this relatively wide-ranging and variable species. After accounting for the range of variation found in *S. varius*, all species are easily identified by plumage, and we found no ambiguity separating these four taxa in museum collections.

Conservation Implications

Seven of the nine taxa described in this complex are endemic to one or a few small islands and were of potential conservation concern. Three of these taxa met our criteria for species recognition, and, because independent evolutionary lineages are widely regarded as important units for conservation (e.g., Ryder 1986; O'Brien and Mayr 1991; Moritz 1994; Vogler and Desalle 1994), we suggest that *S. castaneoventris*, *S. owstoni*, and *S. olivaceus* are obvious candidates for listing, prioritization, and management.

However, although subspecies are recognized by many conservation organizations and often included in protective legislation (Haig et al. 2006), we question the usefulness of retaining *S. v. amamii*, *S. v. orii*, *S. v. sunsunpi*, *S. v. yakushimensis*, and *S. v. namiyei* as named taxa and suggest that their continued recognition could actually mislead conservation efforts

(Zink 2004). We recognize that some morphological characters differed significantly among some of the above subspecies (although most did not) (Appendix). However, *S. varius* is a variable species with clinal geographic variation, and, with a large enough sample size, many significant morphological differences could probably be found among most, if not all, subspecies (as well as countless various combinations of populations distributed along the geographic trends in character variation). The problem is that there is no evidence that these subspecies are evolving independently, that is, evolutionary processes such as mutation, natural selection, and genetic drift are potentially shared.

As Wilson and Brown (1953) pointed out, pervasive gene flow produces discordant patterns of variation among characters, making taxonomic limits inherently subjective and arbitrary. This was reflected in our quantified character distributions; patterns of variation among *S. v. varius*, *S. v. amamii*, *S. v. orii*, *S. v. sunsunpi*, *S. v. yakushimensis*, and *S. v. namiyei* were different for different characters. Alternative subspecies limits could be erected by spotlighting different characters, and characters could be arranged in various combinations to achieve different results. Based on current knowledge, any taxonomic boundaries defined within the geographically variable *S. varius* would be arbitrary. We suggest that conservation efforts not become bound by such inherently subjective divisions.

Conclusions

It is discouraging that recent species delimitation studies have often failed to make taxonomic recommendations (Carstens et al. 2013). This trend is, at least in part, explained by a lack of adequate tools for reassessing some of the characters that formed the basis of many original taxonomic descriptions. Here we have shown how a digital photographic approach to color quantification can aid species delimitation and provide justification for taxonomic revision. Although we focused on an avian example, our general approach to color quantification can be applied without modification to most groups of organisms. This should be especially useful in the diverse range of taxa in which color characters have been featured in taxonomic descriptions; however, these methods can be applied to any taxonomic group that demonstrates variation in color characters. Ongoing advances in tools such as digital photography will continue to enable more comprehensive and more integrative systematic assessments, which should provide better evidence-based justification for effecting taxonomic change.

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.6q44t.

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Appendix

List of morphological differences compiled from original taxonomic descriptions that were reported as distinguishing nine taxa currently recognized in the varied tit complex (*Sittiparus varius*). The taxa *castaneoventris* and *owstoni* were originally described as species; all other taxa were described as subspecies. For each reported character difference, we assessed three aspects of differentiation. First, we assessed whether the character distributions from our quantified measurements overlapped between newly described taxa and the appropriate comparison taxa mentioned in the descriptions. Second, we applied *t*-tests to determine whether reported character differences were statistically significant, again, comparing only described taxa to appropriate comparison taxa. Finally, we determined whether reported character differences were diagnosable in the context of the complex as a whole.

Putative taxon	Description	Reported character difference			Character distributions overlapping?	Statistical significance (*P*-value)	Character diagnostic?
castaneoventris	Gould 1863	Overall size	Smaller than	*varius*	No	0.0001	Yes
owstoni	Ijima 1893	Frontal band[a]	Different color than	*varius*	No	< 0.0001	Yes
		Nape streak	Different color than	*varius*	No	< 0.0001	Yes
		Mantle	Different color than	*varius*	No	0.0002	No
		Breast	Different color than	*varius*	No	< 0.0001	No
		Flanks	Different color than	*varius*	No	NS	No
		Throat band	Different color than	*varius*	No	< 0.0001	Yes
		Overall size	Larger than	*varius*	No	< 0.0001	No
		Bill	Longer than	*varius*	No	< 0.0001	No
		Bill	Thicker than	*varius*	Yes	NS	No
namiyei	Kuroda 1923	Frontal band	Different color than	*varius*	Yes	NS	No
		Nape streak	Different color than	*varius*	Yes	NS	No
		Mantle	Different color than	*varius*	No	0.0002	No
		Breast	Different color than	*varius*	Yes	NS	No
		Tarsus	Longer than	*varius*	Yes	NS	No
		Frontal band	Different color than	*owstoni*	No	< 0.0001	No
		Nape streak	Different color than	*owstoni*	No	< 0.0001	No
		Breast	Different color than	*owstoni*	Yes	NS	No
		Back	Different color than	*owstoni*	No	< 0.0001	No
		Overall size	Smaller than	*owstoni*	No	< 0.0001	No
sunsunpi	Kuroda 1919	Back	Different color than	*varius*	Yes	NS	No
		Black of head	Different color than	*varius*	Yes	NS	No
		Nape streak	Different color than	*varius*	Yes	NS	No
		Mantle	Different color than	*varius*	Yes	NS	No
		Throat	Different color than	*varius*	Yes	< 0.0001	No
		Breast	Different color than	*varius*	Yes	NS	No
		Flanks	Different color than	*varius*	Yes	NS	No
		Overall size[b]	Smaller than	*varius*	Yes	NS	No
		Tail length	Shorter than	*varius*	Yes	NS	No
yakushimensis	Kuroda 1919	Bill	Shorter than	*namiyei*	Yes	NS	No
		Bill	Thinner than	*namiyei*	Yes	NS	No
		Tarsus	Shorter than	*namiyei*	Yes	NS	No
		Tail	Shorter than	*namiyei*	Yes	NS	No
		Frontal band	Different color than	*sunsunpi*	Yes	NS	No
		Breast	Different color than	*sunsunpi*	Yes	< 0.0001	No
		Flanks	Different color than	*sunsunpi*	Yes	NS	No
amamii	Kuroda 1922	Flanks	Different color than	*varius*	Yes	NS	No
		Back	Different color than	*varius*	Yes	0.0003	No
olivaceus	Kuroda 1923	Frontal band	Different color than	*owstoni*	No	0.0004	Yes
		Nape streak	Different color than	*owstoni*	No	< 0.0001	No
		Back	Different color than	*owstoni*	Yes	NS	No
		Overall size	Smaller than	*owstoni*	No	< 0.0001	No
		Bill	Smaller than	*owstoni*	No	< 0.0001	No
		Wing	Shorter than	*owstoni*	Yes	0.0001	No
orii	Kuroda 1923	Frontal band	Different color than	*owstoni*	No	0.0004	No
		Nape streak	Different color than	*owstoni*	No	< 0.0001	No
		Breast[c]	Different color than	*owstoni*	No	< 0.0001	No
		Flanks[c]	Different color than	*owstoni*	Yes	NS	No
		Tarsus	Shorter than	*owstoni*	Yes	< 0.0001	No
		Tail	Shorter than	*owstoni*	Yes	NS	No
		Wing	Shorter than	*owstoni*	Yes	NS	No
		Frontal band	Different color than	*amamii*	No	NS	No
		Overall size	Different than	*amamii*	Yes	NS	No

[a]Following Kuroda 1923, we considered the contiguous area containing the lores, ear-coverts, and sides of neck as a single plumage character that we refer to as the frontal band; color measurements of the frontal band were made at the ear-coverts.
[b]Described as "total length."
[c]Described as "underparts."

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