USING SPECTRAL DATA TO RECONSTRUCT EVOLUTIONARY CHANGES IN COLORATION: CAROTENOID COLOR EVOLUTION IN NEW WORLD ORIOLES

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Abstract.—Carotenoid-based colors are thought to play an important signaling role in many animal taxa. However, little is known about evolutionary changes in carotenoid coloration, especially among closely related species. We used a phylogenetic perspective to examine carotenoid color changes within New World orioles (genus *Icterus*). Oriole color was quantitatively measured using reflectance spectrometry. We found continuous variation from short- to long-wavelength carotenoid colors in extant orioles—perceived by humans as ranging from yellow to scarlet—suggesting that these carotenoid-based colors have evolved as a continuous character. Ancestral state reconstruction suggests that short- and long-wavelength carotenoid colors have evolved independently multiple times, likely from a middle-wavelength ancestor. Although color showed considerable lability, we found a significant amount of phylogenetic signal across the entire genus. This implies that while labile, the colors of closely related taxa tended to resemble each other more than would be expected due to chance. To our knowledge, this is the first study to use quantitative character states derived from reflectance spectra in ancestral state reconstruction. Reflectance spectra provide an unbiased quantitative description of color that allowed us to detect subtle changes among closely related taxa. Using these quantitative methods to score and reconstruct color changes among closely related taxa provides a better understanding of how elaborate animal colors evolve.

Key words.—Ancestral state reconstruction, carotenoid, color, constraint, continuous character, evolution, lability.

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Carotenoid-based colors are a common and widely studied type of elaborate animal coloration. Carotenoids produce many of the red, orange, and yellow colors found in fish, reptiles, and birds (reviewed in Fox 1976; Brush 1990; Hill 2002; McGraw 2006a; but see exceptions in Grether et al. 2001; McGraw et al. 2004; McGraw and Nogare 2005; McGraw 2006b, c). Recently, several behavioral studies in vertebrates have demonstrated female preferences for brighter, more saturated, or longer-wavelength coloration in males (reviewed in Hill 2002, 2006). Physiological studies have begun to elucidate the relationship between individual health and the intensity of carotenoid coloration (e.g., Hill 1991, 2002; Grether 2000; Faivre et al. 2003), and comparative studies have started to examine the influence of factors such as the environment, sensory drive, or species recognition on color (e.g., McNaught and Owens 2002). However, relatively little attention has been paid to the evolutionary history of carotenoid ornaments, especially from a phylogenetic perspective (but see Gray 1996; Hill and McGraw 2004; McGraw and Schuetz 2004).

Most phylogenetic studies that address color evolution have focused on the presence or absence of discrete ornaments, such as carotenoid-based bill coloration or conspicuous epaulets (Johnson 1999; Johnson and Lanyon 2000; Omland and Hofmann 2006), rather than differences in the coloration of particular structures across taxa. The few studies that have attempted to reconstruct evolutionary changes in color define character states in very broad terms, such as red and yellow (Hill and McGraw 2004) or carotenoid and phaeomelanin (Hofmann et al. 2006a). Despite recent advances in the ability to score color quantitatively (due in part to the advent of easy-to-use spectrometers), no studies have attempted to use these quantitative data in ancestral state reconstruction (but see Thorpe 2002; Thorpe and Stenson 2003 for phylogeographic studies). Although broad qualitative col-

or classes may adequately describe coloration in some cases, there are many cases where small differences in color are present, especially among closely related taxa. The ability to detect and quantify these subtle color differences is critical for studies that attempt to reconstruct evolutionary changes in coloration.

New World orioles (*Icterus*) are an ideal genus for investigating carotenoid evolution. Most orioles have carotenoid-based color plumage that ranges from lemon-yellow to scarlet. In addition, Omland et al. (1999) generated a well resolved mtDNA phylogeny for the oriole genus that is also supported by nuclear DNA (Allen and Omland 2003). Omland and Lanyon (2000) used this mtDNA phylogeny to demonstrate pattern convergence in orioles. Omland and Lanyon also found considerable lability in individual pattern elements, even among closely related taxa (also see Kondo and Omland 2004). However, these studies (Omland and Lanyon 2000; Kondo and Omland 2004) focused entirely on pattern (i.e., the location of black, white, and colored plumage on the body) and did not address changes within colored plumage (e.g., yellow to orange or red to yellow). These changes in color are likely controlled by different evolutionary and developmental pathways than those which produce pattern. Pattern in orioles (and many other birds) primarily depends on the location of eumelanin deposition (Theron et al. 2001; Mundy et al. 2004; Mundy 2005; also see Hoekstra and Price 2004). In contrast, carotenoid-based coloration depends on the ability to obtain, absorb, metabolically modify, concentrate, and deposit carotenoid pigments into the feather (Brush 1990; Saks et al. 2003; McGraw 2006a). Furthermore, even a quick survey across the genus *Icterus* reveals that there are many orioles with very similar patterns that differ markedly in color, suggesting that color and pattern can vary independently (see illustrations in Jaramillo and Burke 1999; Allen and Omland 2003, cover).

The proximate mechanisms that produce carotenoid-based colors have important implications for defining color character states and for reconstructing color evolution (Omland and Hofmann 2006). The bright orange plumage of the Baltimore oriole (*Icterus galbula*) is produced by a mixture of carotenoid pigments including canthaxanthin, astaxanthin, doradexanthin, lutein, and canary xanthophylls (Hudon 1991; K. McGraw, pers. comm. 2004). Because color depends on both the types and concentrations of carotenoid pigments present (Saks et al. 2003; McGraw 2006a), a complex mixture of pigments suggests that continuous variation from red to yellow could be generated by increasing or decreasing the ratio of red to yellow carotenoids. Therefore, oriole coloration has the potential to evolve as a continuous, rather than a discrete character, and if supported by the character states of extant taxa (i.e., continuous variation is observed), should be reconstructed as such (Omland and Hofmann 2006). In this situation, quantitative measures of color are important in determining how best to code color characters (discrete vs. continuous) in addition to providing the quantitative raw data necessary for continuous reconstruction.

Thus, we had three main goals in this study. Our first objective was to measure carotenoid color quantitatively across the oriole genus and determine how best to score and reconstruct color evolution. We then wanted to use ancestral state reconstruction to investigate evolutionary changes in carotenoid coloration and test whether oriole coloration appears to show phylogenetic signal (a tendency for closely related taxa to resemble each other). Finally, we wanted to investigate the robustness of our results by examining whether different assumptions of character coding and evolution influenced the reconstruction of ancestral states and the detection of phylogenetic signal.

METHODS

Measurement and Analysis of Reflectance Spectra

We measured reflectance spectra from adult male oriole museum specimens from the Smithsonian National Museum of Natural History, the Delaware Museum of Natural History, and the Field Museum of Natural History. Reflectance spectra were measured from five adult male specimens of each taxon (with the exception of four taxa for which only one to four specimens were available, see Appendix 1 available online at: http://dx.doi.org/10.1554/06-190.1.s1) across six different body regions: the breast, crown, back, rump, belly, and epaulet (a shoulder patch defined as uniformly colored middle and lesser coverts). At least one of these regions is brightly colored (i.e., non-black) in all orioles. However, because of the lability in oriole pattern—regions can be colored, black or white (Omland and Lanyon 2000)—there is no single region that is brightly colored across all orioles. In total, we scored color from 44 oriole taxa (Table 1) for which branch length information was available (Omland et al. 1999). Of those taxa, four had color plumage that was due in whole or in part to phaeomelanins rather than carotenoids (Hofmann et al. 2006a,b) and were excluded from this study.

Reflectance spectra were measured using a spectrometer (Ocean Optics USB 2000, Dunedin, FL) and a full spectrum light source (Ocean Optics PX-2, Dunedin, FL), which provides illumination in the ultraviolet and visible regions of the spectrum. A bifurcated fiber optic cable was used, which allowed for illumination and detection to occur through the same probe. An anodized aluminum cylinder with a 1.27 cm aperture prevented ambient light from entering. All measurements were taken perpendicular to the feather surface and were relative to a Spectralon diffuse white standard (Labsphere, North Sutton, NH) and the dark. Each individual region of plumage was measured in triplicate, from nonoverlapping areas whenever possible. Raw spectral data were binned into one nanometer intervals from 300–700 nm using custom programs (designed by C. Hofmann and T. Cronin), and the triplicate measurements from each body region were averaged.

Defining Color

Color is a perceptual attribute that relates to the shape of a reflectance spectrum (provided that illumination and visual sensitivity are held constant), particularly the regions of the spectrum (wavelengths) where high and low reflectance occur (Wyszecki and Stiles 1982; Endler 1990; Andersson and Prager 2006). Perceived color has multiple dimensions including hue (e.g., red vs. yellow), saturation (e.g., red vs. pink), and brightness (Wyszecki and Stiles 1982; Endler 1990; Cuthill 2006). In this study we focus only on measurements that correspond to the dimension of hue.

Carotenoid-based colors absorb light maximally at short wavelengths (around 400–500 nm), producing spectra that sharply increase in reflectance at middle wavelengths and plateau at longer wavelengths (Bleiweiss 2005; Shawkey and Hill 2005; Andersson and Prager 2006). Thus, carotenoid-based spectra have a distinct shape and are said to resemble step functions (Lythgoe 1979; Bleiweiss 2004, 2005; Andersson and Prager 2006). Carotenoid-based colors also have a secondary peak in the ultraviolet region of the spectrum, which is likely due to the subtractive effect of placing a short-wavelength absorbing pigment on a structural white background (Shawkey and Hill 2005; Andersson and Prager 2006; also see Bleiweiss 2005), and was not considered in this study.

We defined carotenoid-colored plumage as any plumage having a carotenoid spectral shape and a maximum reflectance greater than 10%. These criteria excluded ''white'' plumage, which may be perceived by birds as colorful, but which is produced by structural mechanisms and eumelanin black plumage, which is achromatic and lacks the color elements of hue and saturation. We also excluded colored plumage with a predominant phaeomelanin influence that was readily detectable in the reflectance spectrum (e.g., Hofmann et al. 2006a,b) in which color is produced by a biochemically unrelated class of pigments (Fox 1976; McGraw 2006a,b). These predominantly phaeomelanin-based colors have distinctly different spectral shapes and much longer-wavelength spectral locations that are not continuous with those of carotenoid-based colors. However, we acknowledge that a low concentration of phaeomelanins may be present in some or all of the plumage we scored as carotenoid.

To score carotenoid-colored plumage quantitatively, we determined the wavelength of 50% reflectance ($\lambda R50_{vis}$, here-

TABLE 1. Spectral location of oriole carotenoid coloration (λR50vis). For each taxon the longest wavelength region is in bold. Note that the crown is the longest wavelength body region in 17 of the 23 taxa with colored crowns. Plumage that had lower than 10% max reflectance was considered achromatic (A). *Icterus spurius* and several other orioles have color that is produced by phaeomelanins (P) and were excluded from this study (Hofmann et al. 2006a,b).

| | Spectral Location (nm) | | | | | | | | | | |
	Breast	SE	Crown	SE	Epaulet	SE	Back	SE	Rump	SE	Belly	SE
I. cayanensis cayanensis	A	—	A	—	**530**	5.03	A	—	A	—	A	—
I. cayanensis pyrrhopterus	A	—	A	—	P	—	A	—	A	—	A	—
I. cayanensis periporphyrus	A	—	A	—	P	—	A	—	A	—	A	—
I. chrysocephalus	A	—	533	2.84	**536**	4.16	A	—	521	2.11	A	—
I. chrysater chrysater	531	2.40	**537**	3.04	A	—	530	2.71	528	2.84	528	2.95
I. chrysater hondae	**541**	8.54	538	2.73	A	—	536	1.45	536	2.89	532	2.85
I. nigrogularis nigrogularis	527	0.86	**534**	0.86	522	3.08	532	2.55	528	1.64	527	1.46
I. nigrogularis trinitatis	524	—	**539**	—	518	—	527	—	516	—	524	—
I. leucopteryx leucopteryx	520	2.32	**525**	4.14	A	—	515	1.81	514	1.62	517	1.66
I. auratus	543	3.81	**559**	6.04	A	—	546	5.16	545	4.26	543	4.85
I. mesomelas mesomelas	519	0.92	**538**	1.14	513	0.51	A	—	520	2.11	517	1.12
I. mesomelas salvinii	519	1.50	**538**	0.49	515	1.07	A	—	518	0.51	517	0.51
I. mesomelas taczanowskii	523	—	**537**	—	516	—	A	—	525	—	519	—
I. graceannae	525	0.8	542	2.5	518	2.6	A	—	529	2.9	518	1.2
I. pectoralis pectoralis	539	3.49	**568**	2.60	532	2.32	A	—	546	3.75	538	3.33
I. gularis tamaulipensis	546	3.73	**557**	3.41	540	3.48	A	—	551	3.33	541	4.13
I. gularis yucatanensis	548	4.34	**566**	3.15	543	1.86	A	—	555	3.48	548	2.39
I. gularis gularis	538	1.46	**549**	1.45	531	2.51	A	—	538	1.17	535	1.53
I. pustulatus formosus	540	4.57	**555**	4.66	532	2.73	525	3.17	545	4.55	537	4.17
I. pustulatus sclateri	534	2.30	**556**	3.56	533	2.89	523	1.03	539	2.27	532	2.24
I. cucullatus nelsoni	530	4.64	529	3.48	A	—	A	—	**541**	5.98	520	1.92
I. cucullatus igneus	558	3.58	557	4.28	A	—	A	—	**558**	2.85	539	2.97
I. icterus ridgwayi	543	2.01	A	—	545	1.78	A	—	**548**	1.36	548	1.02
I. jamacaii croconotus	555	3.70	**564**	2.29	548	2.82	562	3.60	555	2.97	547	4.42
I. icterus strictifrons	547	2.60	**565**	0.87	532	5.27	A	—	553	2.72	548	1.66
I. galbula	**555**	1.39	A	—	547	1.22	A	—	**555**	1.56	549	0.60
I. bullockii bullockii	**543**	3.18	A	—	A	—	A	—	540	3.25	540	2.84
I. bullockii parvus	**530**	2.93	A	—	A	—	A	—	527	2.29	521	3.07
I. abeillei	**529**	1.44	A	—	A	—	A	—	A	—	520	0.89
I. spurius	P	—	A	—	P	—	A	—	P	—	P	—
I. fuertesi	P	—	A	—	P	—	A	—	P	—	P	—
I. dominicensis prosthemelas	A	—	A	—	**525**	1.82	A	—	522	1.10	519	3.02
I. dominicensis northropi	517	3.00	A	—	514	5.50	A	—	**519**	0.50	517	0.50
I. dominicensis melanopsis	A	—	A	—	517	1.05	A	—	**519**	0.66	A	—
I. dominicensis dominicensis	A	—	A	—	**521**	1.40	A	—	516	1.57	514	0.66
I. dominicensis portoricensis	A	—	A	—	**523**	2.16	A	—	523	2.15	A	—
I. wagleri	**538**	3.52	A	—	532	2.73	A	—	530	3.04	532	3.28
I. laudabilis	A	—	A	—	**552**	4.18	A	—	539	3.76	541	1.98
I. bonana	P	—	A	—	P	—	A	—	P	—	P	—
I. oberi	P	—	A	—	A	—	A	—	542	6.79	547	9.28
I. graduacauda audubonii	**517**	0.75	A	—	514	1.53	516	0.80	513	1.08	516	0.86
I. graduacauda graduacauda	**516**	1.30	A	—	512	1.80	514	1.39	511	1.69	513	1.25
I. maculialatus	531	5.73	A	—	**542**	8.93	A	—	523	2.52	519	1.93
I. parisorum	514	0.58	A	—	513	0.87	A	—	**516**	0.86	514	0.71

after referred to as ''spectral location'' in the text) by finding the reflectance midpoint (R50) between the point of maximum and minimum reflectance (Pryke et al. 2001; Andersson and Prager 2006) in the visible (400–700 nm) region of the spectrum (to avoid any influence from the second ultraviolet peak). We then determined the wavelength (to the nearest nanometer) at which this 50% value occurred (Fig. 1). Therefore, the spectral location (λR50$_{vis}$) defines the color of carotenoid-based plumage in a manner that is independent of any particular visual system, but which is still generally related to how visual systems perceive color (also see discussions in Pryke et al. 2001; Bleiweiss 2004, 2005; Andersson and Prager 2006). To the human visual system, carotenoid colors having shorter-wavelength λR50$_{vis}$ values appear more

yellow, and as wavelength increases they become progressively redder (Bleiweiss 2004).

Reconstructing Color

Spectral location values from all orioles were plotted and examined for continuity (by determining whether there were gaps in spectral location). Spectral location was then reconstructed onto the previously published mtDNA phylogeny, with branch lengths scaled to molecular distance (e.g., fig. 7 in Omland et al. 1999). The branch lengths of terminal taxa were adjusted using the ultrametricize function in Mesquite (Maddison and Maddison 2004) to ensure that terminal taxa had equal divergence distances from their common ancestors
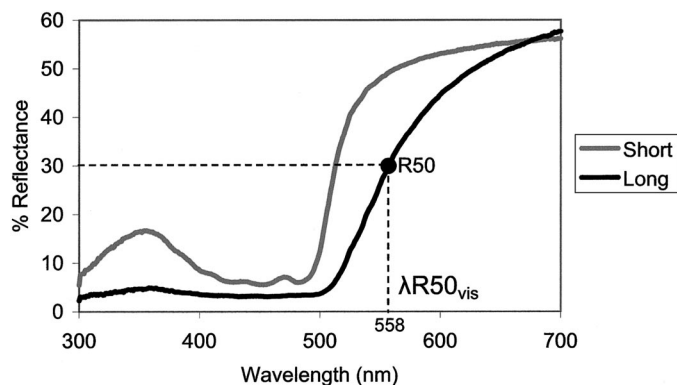
FIG. 1. Examples of short- (gray) and long-wavelength (black) carotenoid reflectance spectra. All carotenoid reflectance spectra have this same general shape characterized by a minor peak in the UV, low reflectance at short wavelengths, a rapid increase in reflectance at middle wavelengths (the location of which determines the perceived color), and a plateau at longer wavelengths (Bleiweiss 2004). The location of the reflectance midpoint (R50) and spectral location ($\lambda R50_{vis}$) are also illustrated for the long-wavelength color. Humans generally perceive carotenoids that have longer-wavelength spectral locations as redder and shorter-wavelength spectral locations as more yellow.

(Mesquite simply stretches the branch lengths of terminal taxa). This makes terminal branches more ''clocklike'' (i.e., equal in length) and was performed to prevent an under/overestimation of evolutionary change on unequal terminal branches.

We used the Continuous-character Model Evaluation and Testing (CoMET) program (Oakley et al. 2005; Lee et al. 2006) to determine which model of evolution best fit our data. When running the program we used the default punctuation asymmetry threshold of 100. CoMET uses maximum likelihood to examine how well nine different evolutionary models fit the tree topology and character data and then uses Akaike Information Criteria (AIC) to determine which model is the best fit. For all body regions a punctuated average/equal model was favored (Appendix 2 available online at: http://dx.doi.org/10.1554/06-190.1.s2). In this model one daughter node is fixed (i.e., retains the parent state) while the other is allowed to vary. Linear parsimony best corresponds to the punctuated average/equal model. Thus, we used linear parsimony implemented in Mesquite to reconstruct spectral location (Maddison and Maddison 2004). Note that continuous methods in Mesquite do not allow for missing data. Therefore, taxa having achromatic plumage for a particular body region were not included in the reconstruction for that body region.

Testing Phylogenetic Signal

We used the MatLab-based continuous character permutation program PHYSIG (T. Garland, available upon request at: tgarland@citrus.ucr.edu) to test whether the carotenoid-based color of each body region had a significant amount of phylogenetic signal (Blomberg et al. 2003). PHYSIG compares the ''fit'' (defined as the mean squared error, MSE) of the observed character states over a given tree (using a generalized least squares approach) to a random distribution of

MSEs generated by permuting the character states over the same tree (Blomberg et al. 2003). We used the default value of 1000 permutations. PHYSIG also calculates the K statistic, a descriptive statistic that describes the strength of phylogenetic signal relative to a Brownian process occurring over the same tree. K values less than one indicate that phylogenetic signal is weaker than a Brownian process, whereas values greater than one indicate that the signal is stronger (Blomberg et al. 2003). PHYSIG requires a variance covariance distance matrix as well as a tip data file, which were generated using the programs PDIST and PDAP, respectively (T. Garland, available upon request).

Testing Assumptions

Different assumptions of character coding and character evolution can alter the reconstruction of ancestral states (Omland 1997, 1999; Omland and Hofmann 2006). To test whether our findings were sensitive to such assumptions, we reconstructed breast spectral location using squared-change parsimony. We also discretenized breast spectral location into three character states. We classified carotenoid spectral locations falling between 510–530 nm as short-, spectral locations between 530–550 nm as middle-, and spectral locations between 550–570 nm as long-wavelength character states. We then reconstructed breast coloration as an ordered character using equally weighted parsimony in MacClade (Maddison and Maddison 2000) and using maximum likelihood (Schluter et al. 1997) with a one parameter Markov k-state model in Mesquite (Maddison and Maddison 2004). We also used MacClade to calculate three descriptive statistics: the retention index (RI), the consistency index (CI), and the number of changes in the reconstruction. To obtain a measure of phylogenetic signal from the discrete ordered parsimony reconstruction, we used the ''shuffle'' function in MacClade to redistribute character states randomly across the phylogeny 100 times (Maddison and Slatkin 1991; Omland and Lanyon 2000). We then compared the number of changes in the randomly generated distribution to the observed number of changes in the reconstruction of breast coloration.

RESULTS

Color Data

We found considerable variation in oriole coloration across taxa. Spectral location appeared to vary continuously across all wavelengths. We found no evidence of discrete differences, such as gaps or jumps, in spectral location measurements. Rather, spectral location appeared to vary continuously with considerable overlap between taxa in many cases (Fig. 2a,b). The spectral location of oriole body regions ranged from 511 to 568 nm (Table 1). The crown had the longest wavelength spectral location of all body regions on average (547 nm), as well as the single longest wavelength measured (568 nm, *I. pectoralis pectoralis*) (Table 2). The epaulet, belly, and back had the shortest average wavelengths (528, 529, and 530 nm respectively) (Table 2). The back had the largest range of variation within a single body region (49 nm), while the belly had the least variation (35 nm) (Table 2).
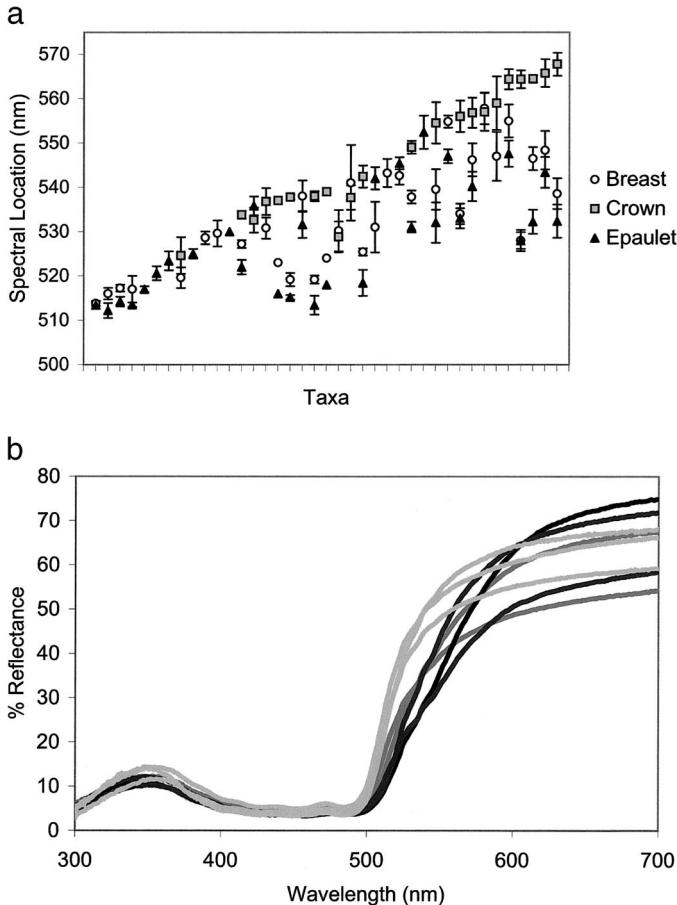
a

b

FIG. 2. (a) Rank plot illustrating the continuous variation in spectral location observed across taxa for three representative body regions. The spectral locations of the breast, crown, and epaulet are plotted vertically along the y-axis. Each tick on the x-axis represents a different taxon (note that not all body regions are colored in all taxa). Bars represent SE. Other body regions showed a similar pattern, but for clarity were not included. (b) Reflectance spectra from the breast of all members of clade B (see Fig. 3) illustrating how spectra varied across taxa. Each spectrum is a composite of individuals from one taxon.

TABLE 2. Summary of oriole color measurements for each body region. Note that on average the crown had a much longer-wavelength spectral location (bold).

	n	Average (nm)	Min (nm)	Max (nm)	Range (nm)
Breast	31	533	514	558	44
Crown	21	**547**	525	568	43
Epaulet	29	528	512	552	40
Back	11	530	514	562	49
Rump	36	532	511	558	47
Belly	34	529	513	549	35

(<5 nm), *I. nigrogularis nigrogularis* and *I. n. trinitatis, I. graduacauda audubonii* and *I. g. graduacauda,* and *I. mesomelas salvinii* and *I. m. taczanowskii* (Fig. 3). Although the crown had the longest wavelength spectral location on average, crown color showed a similar pattern of independent gains of short- and long-wavelength spectral locations (Fig. 4). We found no evidence for directional evolution towards long- (or short-) wavelength colors across taxa.

Phylogenetic Signal

We found that closely related taxa tended to have colors that were more similar than expected due to chance. The permutation analysis in PHYSIG suggested that a significant ($P < 0.05$) amount of phylogenetic signal was present across all body regions (Table 3). Even the back, which was only colored in 11 taxa and had the greatest range of spectral location (Table 2), showed a statistically significant amount of phylogenetic signal (Table 3). All body regions had K values less than one (0.31, belly–0.75, epaulet) except for the back, which had a K value of 1.12 (Table 3). However, the back also displayed lability with multiple gains of short- and long-wavelength colors among the 11 taxa. Although not shown, we obtained similar results when all branch lengths were scaled to one (corresponding to a speciational model).

Testing Assumptions

When we reconstructed breast color as a discrete character using squared-change parsimony, ordered parsimony, and maximum-likelihood methods, we obtained ancestral state reconstructions similar to those produced using linear parsimony (Fig. 6a). Some differences between methods did occur. For example, squared-change parsimony tended to favor more intermediate values (Fig. 6b) than linear parsimony (Fig. 6a). Linear, squared-change and ordered parsimony all favored a middle-wavelength carotenoid as the ancestral state for clade B, whereas maximum likelihood indicated a high degree of uncertainty was present (Fig. 6c). Also, discretenizing the data (Fig. 6c) made there appear to be a much greater difference in color between *I. graceannae* and *I. pectoralis* than when they were treated as continuous characters (Fig. 6a,b). However, all methods of reconstruction suggested the same general pattern of multiple independent gains of short- and long-wavelength carotenoid colors.

When breast spectral location was reconstructed as a discrete ordered character, the consistency (CI) and retention indices (RI) were 0.18 and 0.36 (both values range from 0

Ancestral State Reconstruction

Ancestral state reconstruction revealed that both long- and short-wavelength colors repeatedly evolved (e.g., Figs. 3–5) within orioles across all body regions (the belly, epaulet, and back reconstructions had similar patterns and are not shown). For example, breast spectral locations of 550 nm or greater and 520 nm or less appear to have evolved independently at least three times (Fig. 3). The ancestral spectral location was inferred as a middle-wavelength (relative to the maximum and minimum values found in extant taxa) for all body regions (e.g., Figs. 3–5). In some cases sister taxa had very different spectral locations while in other cases sister taxa had very similar spectral locations. For example, breast spectral locations differed considerably (>25 nm) among two pairs of sister taxa, *I. galbula* and *I. abeillei* and *I. cucullatus igneus* and *I. c. nelsoni.* However, there were also three pairs of sister taxa that had very little difference in breast color
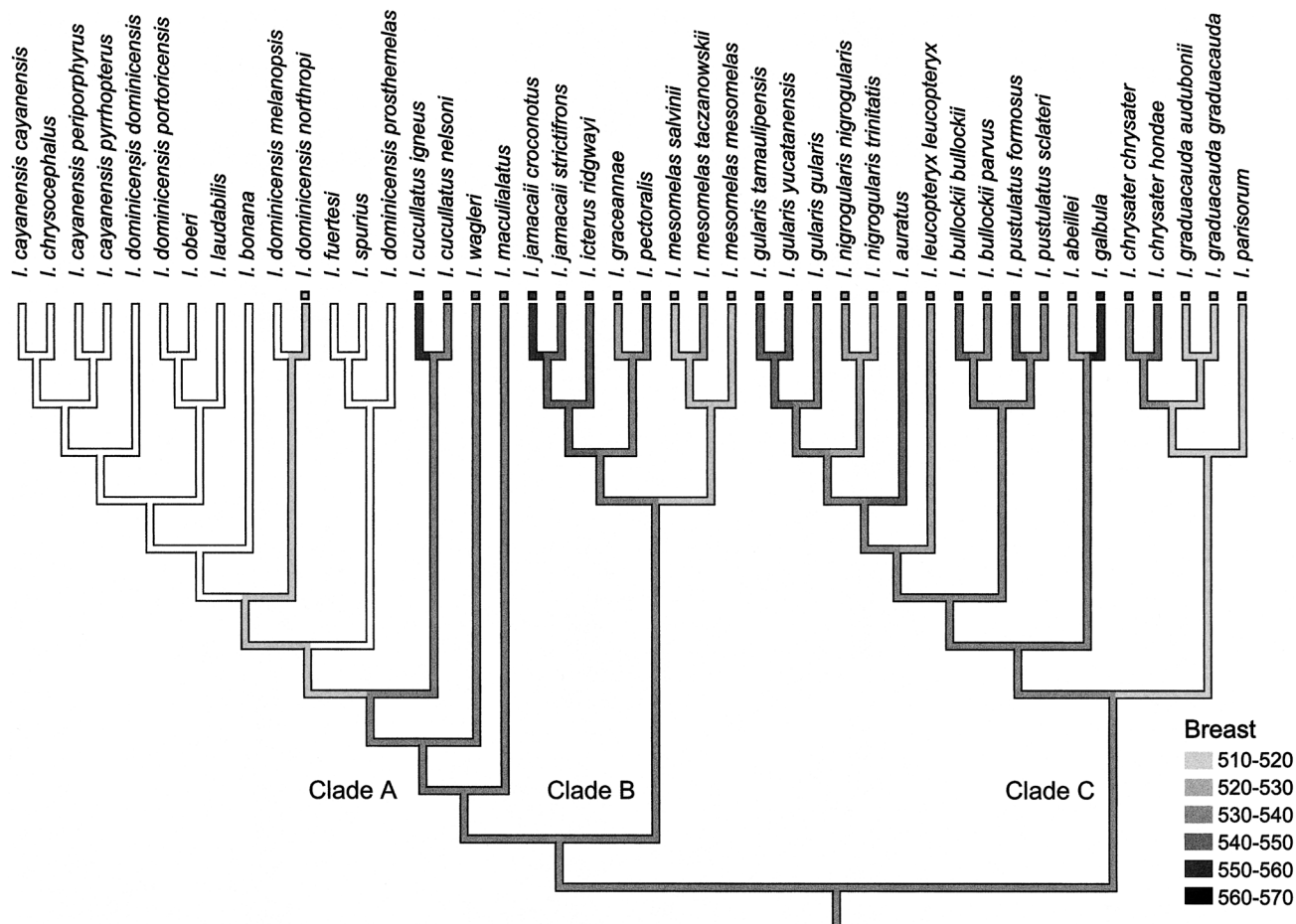
FIG. 3. Ancestral state reconstruction of breast spectral location as a continuous character. Longer-wavelength spectral locations are darker. Spectral location was reconstructed using linear-change parsimony in Mesquite (Maddison and Maddison 2004). We used the complete phylogeny to allow comparisons across body regions and to illustrate how color changes occur in relationship to taxa having achromatic or phaeomelanin based plumage (white branches). These taxa are for illustrative purposes and were not included in any of the analyses. Nodes having a range of values are illustrated with their minimum state (Phylogeny is from Omland et al. 1999).

to 1, see discussion). The discrete ordered reconstruction suggested that 11 steps (changes) had occurred. Randomly shuffling breast color across taxa and reconstructing it as an ordered character produced a tree with 11 steps or fewer 10% of the time ($P = 0.10$). Although this value is not statistically significant, it suggests that some degree of phylogenetic conservatism is present.

DISCUSSION

We found that oriole color varied continuously from short to long wavelengths (Fig. 2a,b). There were no gaps in spectral location that would allow color to be discretenized in any nonarbitrary fashion (e.g., Price and Lanyon 2002). The continuous variation that we documented agrees with previous findings that color can be a complex character that not only depends on the identity of the pigments present, but also their concentrations and relative ratios. Although single-pigment based systems do exist, our data (and the published biochemical data) strongly suggest that carotenoid-based coloration in New World orioles likely evolved as a continuous character and is best reconstructed as such.

Lability and Constraint

We found considerable variation in carotenoid coloration within New World orioles. Long- and short-wavelength carotenoid colors appear to have arisen multiple times across all body regions (Figs. 3–5). We also found several examples where closely related sister taxa, such as I. galbula and I. abeillei, have large color differences that appear to have evolved rapidly over short time periods. In other cases, sister taxa estimated to have greater divergence times, such as the two I. nigrogularis subspecies, have very similar colors (Fig. 3, note that branch lengths are not scaled; also see Omland et al. 1999).

Although oriole color appears quite labile, as suggested by Figures 3–5 and the low values of the K statistic, permutation tests suggested that a significant amount of phylogenetic signal is still present across all body regions (Table 3). Our results suggest that whereas individual oriole colors have the potential to evolve rapidly (i.e., some closely related taxa have very different colors), when color change is examined across the entire genus related taxa tend to resemble each other more than would be expected due to chance. Thus, while
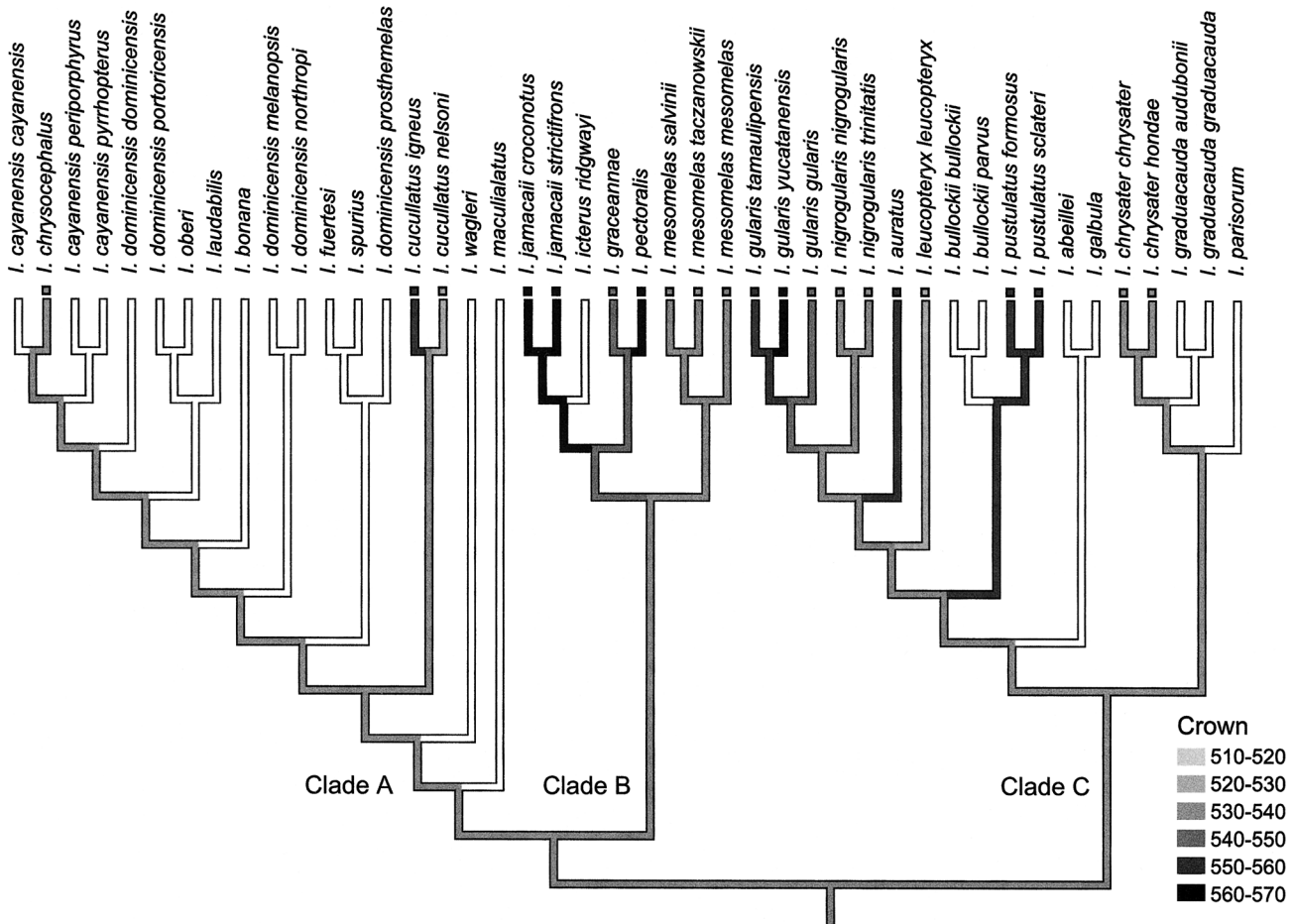
FIG. 4.   Ancestral state reconstruction of crown spectral location as a continuous character. Although on average the crown tended to have a longer-wavelength spectral location than any other body region, there still appeared to be multiple gains of short- and long-wavelength crowns (see Fig. 3 legend for methods of reconstruction).

present, the phylogenetic signal that we observed does not appear to limit color evolution. We follow Blomberg and Garland's (2002) use of the conservative term phylogenetic signal (as opposed to phylogenetic constraint) because it suggests that closely related taxa tend to resemble each other without implying a causative mechanism for this tendency (also see Blomberg et al. 2003).

These repeated gains of both long- and short-wavelength colors do not suggest a directional change from short- to progressively longer-wavelength colors across taxa (also see Hill and McGraw 2004; McGraw and Schuetz 2004). Rather, our ancestral state reconstructions suggest that the oriole ancestral state was a middle-wavelength color with repeated increases and decreases in spectral location (but see discussion below). Although more biochemical analyses are necessary across the oriole genus, our findings suggest the intriguing possibility that a mixture of some or all of the carotenoid pigments found in extant taxa were already present in an ancestral oriole. This mixture of pigments agrees with the reconstructed middle-wavelength ancestral state and would account for the observed lability and phylogenetic signal. However, one alternative is that combinations of different carotenoids produce similar colors across species

(through independent gains of pigments). Although less parsimonious, such a scenario would also explain the observed character states and cannot be completely ruled out using our methods. Thus, future biochemical analyses could reveal interesting evolutionary changes in the carotenoid pigments of these or other taxa.

Assumptions of Ancestral State Reconstruction

Our continuous analyses suggested that middle-wavelength colors were the most likely ancestral states for all body regions (Figs. 3–5). However, we emphasize that these results should be interpreted with caution. The lability that we observed among terminal taxa increases the uncertainty at deeper nodes (Cunningham et al. 1998; Omland 1999). Nevertheless, no aspect of our results suggests a directional evolutionary trend across the genus or contradicts the likelihood of an ancestor with intermediate wavelength coloration—intermediate wavelength taxa predominate and are distributed quite uniformly across all clades of the tree (Figs. 3–5).

The evolutionary model that best fit our data was a punctuated equal model. This model agreed with the rapid changes that we observed among closely related taxa and suggested
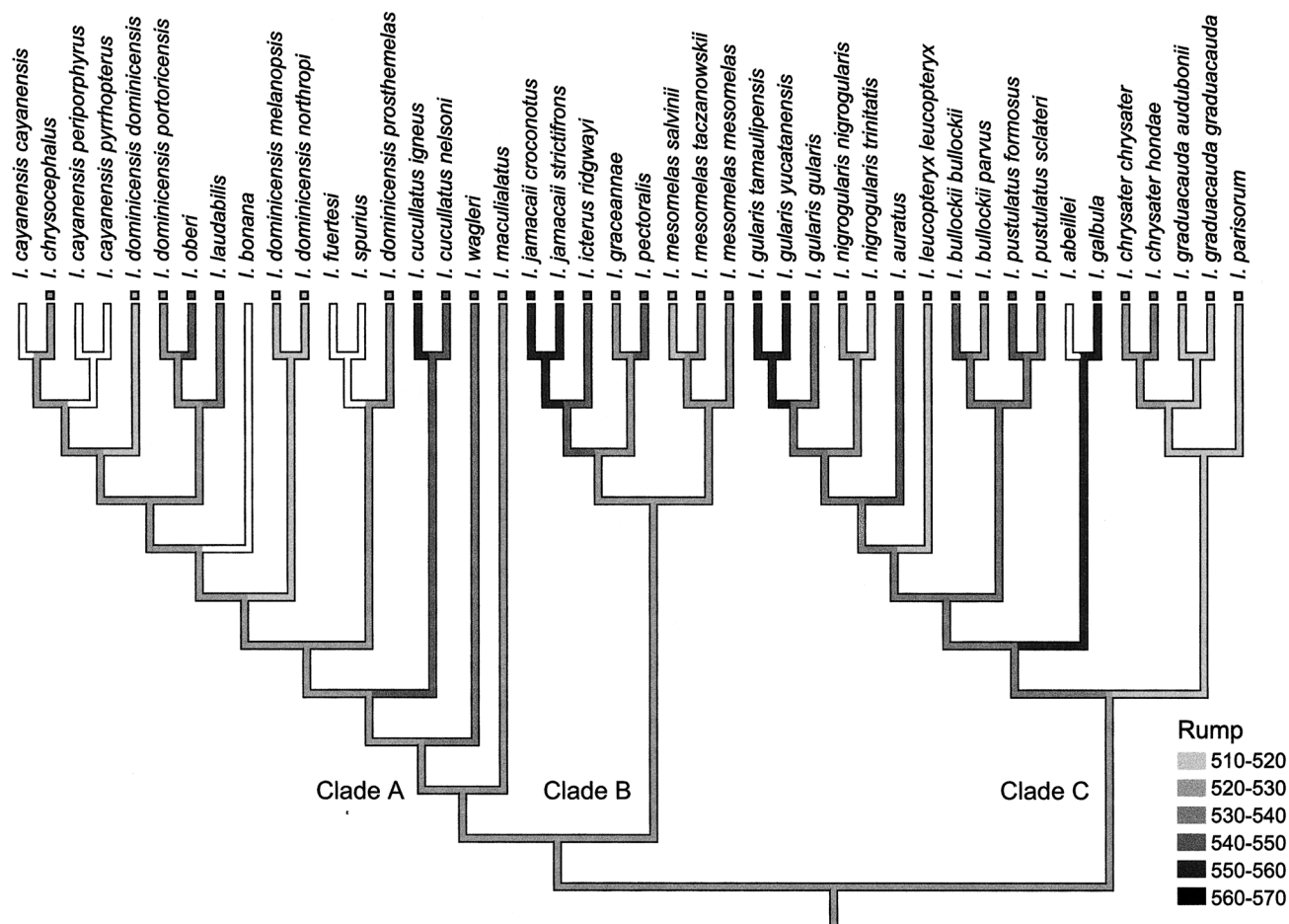
FIG. 5. Ancestral state reconstruction of rump spectral location as a continuous character. This reconstruction includes several taxa in clade A that did not have colored breasts or crowns (see Fig. 3 legend for methods of reconstruction).

that linear parsimony (rather than squared-change parsimony) is the best method to reconstruct our data. However, our findings were relatively robust to the use of different methods of character coding and reconstruction. When color was reconstructed as a continuous character using linear parsimony, the character state at many deeper nodes was inferred to be a middle-wavelength color (Figs. 3–5). Squared-change parsimony, ordered parsimony, and maximum likelihood generally tended to support these inferences, although maximum

TABLE 3. Output from PHYSIG for the body regions examined in this study. All body regions had significant phylogenetic signal. K is a descriptive statistic describing the strength of signal relative to Brownian motion. P values are the probability of producing a MSE equal or less than the observed MSE by chance, based on 1000 permutations in PHYSIG (Blomberg et al. 2003).

	K	Observed MSE	P
Breast	0.35	200.58	0.02
Crown	0.49	189.67	0.02
Epaulet	0.75	84.76	<0.001
Back	1.12	121.50	0.01
Rump	0.58	169.60	<0.001
Belly	0.31	234.66	0.05

likelihood indicated that in many cases a great deal of uncertainty was present (Fig. 6). Other minor differences occurred due to the different assumptions that each method makes (Omland 1997; Martins 1999; Webster and Purvis 2002). For example, squared-change parsimony tended to favor more intermediate spectral locations because it attempts to minimize change over branch lengths. Also, discretenizing the data resulted in the appearance of greater differences between some taxa whose spectral locations were quite similar but fell on either side of the arbitrarily set character states (Fig. 6). Despite these differences at a few ancestral nodes, all methods of reconstruction (linear parsimony, squared-change parsimony, discrete ordered parsimony, and maximum likelihood) suggested the same overall pattern of evolution (Fig. 6). Thus, carotenoid-based colors appear to show considerable lability, multiple gains of short- and long-wavelength colors, and no evidence of directional evolution regardless of the assumptions we used to code character states or the methods used to reconstruct evolutionary changes.

When we reconstructed breast coloration using discrete ordered parsimony, the consistency (CI) and retention (RI) indices were relatively low, reflecting the high degree of color lability and homoplasy. When we tested for phylogenetic
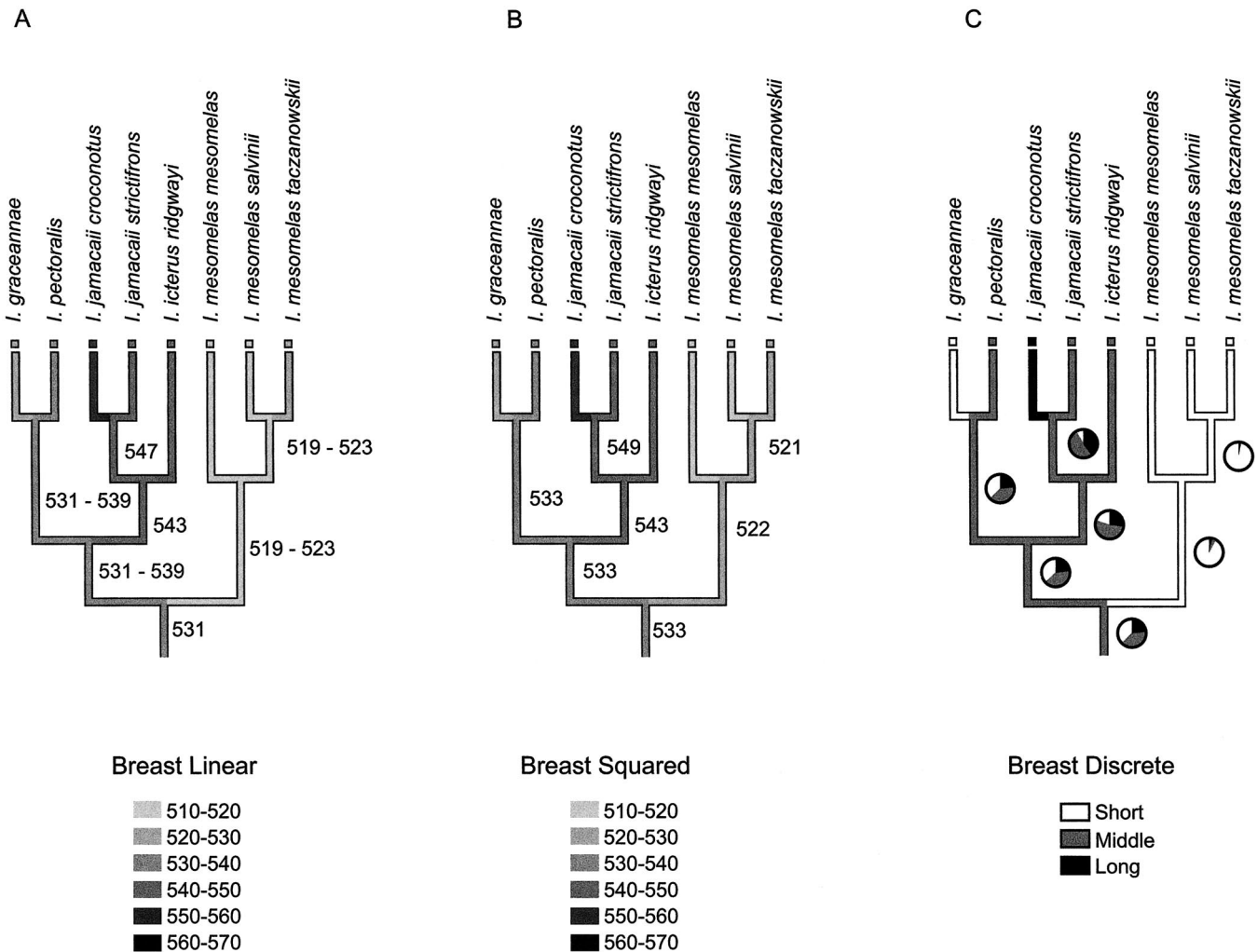
FIG. 6. Ancestral state reconstruction of the breast from all species in clade B using (a) linear parsimony, (b) squared-change parsimony, and (c) two discrete methods: ordered parsimony and maximum likelihood. For the ordered parsimony and maximum likelihood, breast spectral locations were discretenized at 20 nm intervals into short- (510–530 nm), medium- (530–550 nm), and long-wavelength (550–570 nm) character states. Branches are shaded with the most parsimonious character state obtained using ordered parsimony. Pie diagrams indicate relative likelihood values at the nodes obtained using a Markov k-state model in Mesquite (Maddison and Maddison 2004). Although not drawn to scale here, branch lengths were included in the maximum-likelihood and squared-change parsimony reconstructions.

signal by comparing the observed number of changes (steps) in the parsimony reconstruction to the amount of change in a distribution of randomly shuffled character states, phylogenetic signal was still observed. However, this amount of phylogenetic signal was not statistically significant. Two possible explanations could account for these differences in sensitivity between continuous and discrete methods. One is that the PHYSIG GLS test for phylogenetic signal in continuous characters takes into account branch lengths, whereas the parsimony method used for the discretenized data does not. The other reason is that reconstructing a continuously evolving character as a discrete, rather than a continuous, character will cause errors when estimating the amount of change that occurs (Omland and Hofmann 2006). These errors will happen at two steps: when arbitrarily discretenizing data (taxa having a range of values will be considered to have the same character state, and taxa having very similar values that fall

just outside that interval will be considered to have different character states); and when reconstructing the changes themselves (discrete methods assume that changes occur in a single step, continuous methods assume many small steps, also see Omland and Hofmann 2006). These results suggest that coding a continuous character as discrete might decrease the ability to detect phylogenetic signal.

Similarities between Color and Pattern Evolution

Our carotenoid color findings have several similarities with the results of Omland and Lanyon's (2000) investigation of oriole pattern. Although produced by different proximate mechanisms, carotenoid coloration and pattern show similar evolutionary tendencies. Both color and pattern are highly labile with individual pattern elements, such as black foreheads, being repeatedly gained and lost (see fig. 4 in Omland

and Lanyon 2000), and different colors, such as long-wavelength carotenoids, arising multiple times. However, these evolutionary phenomena appear to be independent. Large differences in both color and pattern may be found between some pairs of sister taxa, such as *I. galbula* and *I. abeillei*. Other pairs of sister taxa, such as *I. cucullatus nelsoni* and *I. cucullatus igneus*, have almost identical patterns but large color differences. Finally, as with pattern, our results suggest that color is not an ideal character to use when constructing phylogenies. Although carotenoid coloration appears to have a significant amount of phylogenetic signal, there also appears to be a considerable amount of homoplasy, with short- and long-wavelength colors arising independently multiple times. However, the high lability observed among closely related taxa does suggest that quantitative color measurements may be useful in delineating species boundaries.

Conclusions

To our knowledge, this is the first study to use quantitative characters derived from reflectance spectra in ancestral state reconstruction. Spectrometric methods have become increasingly important in behavioral studies because they are quantitative and not subject to the biases of the human (or any) visual system (Bennett et al. 1994). The quantitative detail provided by spectral data may offer further insights into color evolution by allowing the investigation of small color differences among closely related taxa. Furthermore, the methods used in this study need not be limited to carotenoids or signal evolution. With careful consideration, they could also be used to reconstruct other aspects of coloration such as sexual dichromatism, crypsis, or conspicuousness. Thus, the techniques used in this study are applicable to all animals and even plants.

Although many intraspecific behavioral studies in vertebrates suggest that females may favor longer wavelength male coloration, we did not observe any evidence of directional color changes across oriole species. Instead, our results suggest that the oriole ancestral state was an intermediate wavelength color (i.e., orange) and that there were multiple independent gains of longer- (redder) and shorter-wavelength (more yellow) colors. This result is not surprising for several reasons. First, little behavioral work has been done in orioles, and it is not known whether females even favor longer-wavelength colors. Second, these two issues deal with different levels of evolution. One examines behavior within a species whereas the other examines changes across species. Although female preference might drive color to change within a single species over evolutionary time, there is no reason that this change would occur in a way that suggests a progression across extant taxa, or that females of other species would have similar preferences. Rather, if a female preference for redder plumage arose, then all subsequent lineages would be expected to have redder plumage regardless of whether they are basal or derived (provided there are no physiological/environmental limitations).

Finally, this study demonstrates some of the challenges that arise when deciding which assumptions should be used to reconstruct spectral data (also see Omland and Hofmann 2006). Although our measurements from extant taxa suggest that color should be reconstructed as a continuous character, we can think of many cases when the data might not support such a reconstruction. One obvious example would be if two separate pigments are known to produce color plumage and mixtures are not found. For example, we previously found that several orioles appear to have color that is produced predominantly by phaeomelanins (Hofmann et al. 2006a,b). We could have generated spectral location ($\lambda R50_{vis}$) values for these phaeomelanin-based colors and included them in our analyses. However, scoring carotenoid and phaeomelanin colors on the same continuous character scale is not justified by the data—they are separate pigment classes with entirely different spectral shapes and discretely different spectral locations (generally over 600 nm, data not shown). Observations from extant taxa combined with our knowledge of the proximate mechanisms make it clear that in this case such an approach would be naive and would result in misleading reconstructions (Omland and Hofmann 2006). However, when careful consideration is given to the methods used to reconstruct color, the use of quantitative spectral data will continue to provide valuable new insights into color evolution across birds and a wide range of other taxa.

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