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Research paper

Phylogenetic signal in the skull of cowbirds (Icteridae) assessed by multivariate and cladistic approaches

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ABSTRACT

Closely related species are expected to resemble each other because of inheritance of features from their common ancestors, which are therefore said to carry phylogenetic signal. Cowbirds (*Molothrus*) are icterids well known from being interspecific brood parasites, but their taxonomy and evolutionary relationships have varied considerably based on morphology alone. In turn, these relationships became unsupported by molecular phylogenies, lending lower value to morphological data in general. However, the osteology of cowbirds has not yet been studied under a quantitative phylogenetic framework and it is uncertain whether their skulls carry phylogenetic signal. In order to test this, we assembled a data matrix, including continuous and discrete characters of the skull of cowbirds and allies, and analyzed it with two complementary approaches under the evolutionary framework provided by molecular phylogenies. We first took a multivariate approach, exploring the occupation of a phylomorphospace based on skull data and estimating the amount and significance of phylogenetic signal by calculation of the K_{mult} statistic. The second approach relied on a Maximum Parsimony optimization of characters on a scaffold tree. Our results indicate that, although some homoplasy exists, the skull of cowbirds and allies carries significant phylogenetic signal and provides useful characters to diagnose *Molothrus* and other still poorly diagnosed clades. This first comparative approach is promising and opens the possibility of integrating data from fossils, while encouraging further osteological analyses in cowbirds and other icterids.

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1. Introduction

The assumption that the phenotype of organisms carries signal of their evolutionary history lies at the core of taxonomic and systematic studies based on morphology. Closely related species are expected to resemble each other because of inheritance of features from their more recent ancestors and those features are therefore said to carry phylogenetic signal (Blomberg et al. 2003; Adams & Collyer 2019). Yet, under many evolutionary scenarios certain parts of the phenotype might not truly reflect common ancestry, for instance due to convergence linked to common functional demands in distantly related taxa, and are said to show homoplasy (Hall 2013). Unquestionably, the ongoing molecular

'revolution' in avian phylogenetics (e.g., Prum et al. 2015; Oliveros et al. 2019) has revealed that several morphology-based taxonomic and systematic arrangements were grounded on homoplastic features, including that of cowbirds and their kin (Lanyon 1992; Lanyon & Omland 1999; Powell et al. 2014; Remsen et al. 2016).

Cowbirds (*Molothrus* Swainson, 1832 in Swainson & Richardson, 1831) comprise five (or six, if *Molothrus armenti* Cabanis, 1851 is recognized) species of Icteridae that are widely distributed across the Americas and are well known from being obligated interspecific brood parasites (Fraga & Bonan 2019; Remsen et al. 2019). Based on morphology alone the taxonomic content of the genus *Molothrus* has varied considerably. For instance, the Giant Cowbird, *Molothrus oryzivorus* (Gmelin, 1788), and the Bronzed Cowbird, *Molothrus aeneus* (Wagler, 1829), have formerly been classified in separate monotypic genera (Hellmayr 1938; Beecher 1951; Webster 2003); whereas baywings (now in their own genus *Agelaioides*) have long

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been considered as part of *Molothrus* (see Johnson & Lanyon, 1999; Remsen et al., 2019). These taxonomic arrangements regarding cowbirds and other icterids became unsupported by molecular phylogenies (e.g., Lanyon 1992; Johnson & Lanyon 2000; Powell et al. 2014), which has led to undervaluing of morphological data in general, due to it being considered poorly informative of their phylogeny (Powell et al. 2014).

Notably, although we have extensive knowledge on several aspects of cowbird biology based on hundreds of ecological, behavioral, and evolutionary studies on model species (for comprehensive reviews see Soler 2017; Rebores et al. 2019; Sherry & Guigueno 2019), cowbird morphology in general has received comparatively little attention, with most work focused on plumage (e.g., Omland & Lanyon 2000; Eaton 2006; Friedman et al. 2011), and the scarce skeletal data published to date, mostly of their skulls, has not yet been investigated from a quantitative comparative viewpoint (e.g., Beecher 1951; Webster 2003; Oswald & Steadman 2011, 2015). Björklund (1991) performed a cladistic analysis of grackles with skull data, but included only *Molothrus ater* (Boddaert, 1783) among the outgroup taxa. Such an approach has been hampered not only by the paucity of skeletal data, but also by the unstable systematic arrangement and changing perceived relationships of cowbirds over the last century (Hellmayr 1938; Blake 1968; Remsen et al. 2019). As a consequence, it is yet unknown whether the skeleton of cowbirds carries phylogenetic signal and our present understanding of their evolutionary relationships still lacks support from osteology. This, in turn, prevents integration of skeletal data in evolutionary studies including that from fossils (e.g., Oswald & Steadman 2011, 2015). The robust phylogenetic consensus on icterid relationships, including cowbirds, recently reached by molecular studies (Powell et al. 2014; Remsen et al. 2016) provides the opportunity to explore this issue.

With this in mind, we here quantitatively analyze data from the skull of cowbirds in an explicit evolutionary framework in order to test its actual phylogenetic signal. To do this we employ different, but complementary, multivariate and cladistic approaches. We find that the skulls of cowbirds carry significant phylogenetic signal and we advanced a few potential osteological synapomorphies for different clades.

2. Materials and methods

2.1. Institutional abbreviations

AMNH: American Museum of Natural History, New York, USA; FMNH: Field Museum of Natural History, Chicago, USA; MACN: Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires, Argentina; UF, Florida Museum of Natural History, University of Florida, Gainesville, USA.

2.2. Taxon sampling

We surveyed the five species of *Molothrus* currently recognized by the South and North American Classification committees of the American Ornithological Society (Remsen et al. 2019). Several dry adult specimens of each species were examined, sampling different subspecies whenever possible, totaling 38 dry specimens of *Molothrus* (Supplementary data). Outgroup taxa include species representing major groups across Icteridae (sensu Remsen et al. 2016; the use of Cacicinae instead of Cassicinae follows Schodde & Remsen 2016) and closely related families (Supplementary data). Taking into account the phylogenetic relationships of cowbirds within Icteridae (Powell et al. 2014; Remsen et al. 2016) our outgroup sampling emphasized representation of agelaiines (i.e., grackles and allies), including cowbird-like taxa such as the Austral

Blackbird, genus *Curaeus* Sclater 1862, and the Greyish Baywing, *Agelaioides badius* (Vieillot, 1819), the latter of which has been treated as part of *Molothrus* until recently (e.g., Webster, 2003).

2.3. Character sampling and anatomical terminology

We assembled an osteological data matrix including both continuous and discrete characters of the cranium and lower jaw. Continuous characters derived from those previously considered by Webster (2003) in his phenetic approach to icterid systematics and consist of seven standardized ratios representing proportions of distinct skull structures (Supplementary data). Calculation of these ratios relied on linear measurements of the skull and lower jaw. Since some structures are extremely small and in some cases do not offer anatomical buttresses on which anchor a caliper, measurements were taken digitally from photographs of dry specimens using ImageJ v. 1.48 (Schneider et al. 2012). Photographs of each view were taken in a standardized way to reduce the effects of parallax. It has been demonstrated that digital measurements from photographs of well-oriented specimens are as precise as, or even more than, caliper-based measurements taken manually (Lires et al. 2016). When more than one specimen per species was available, we scored means of each ratio pooling data from females and males (we did not note marked sexual dimorphism in any particular character). In addition, neomorphic and transformational variation across species was partitioned into 23 discrete characters of the cranium and lower jaw (Supplementary data), which were built following the recommendations of Sereno (2007). Hereafter, specific character-states are denoted by the respective character and state numbers separated by a colon; for instance, 3:1 indicates state 1 of character 3 (see Supplementary data for character list). We followed the osteological nomenclature of Baumel & Witmer (1993), with most Latin terms translated into English vernacular. Some terms that have been widely used in describing the cranial anatomy of icterids or other nine-primaried oscines (e.g., Beecher 1951; Richards & Bock 1973; Webster 2003) were also used.

2.4. Phylogenetic signal

We estimated the phylogenetic signal in the skull of cowbirds and allies using the resultant data matrix of 30 characters scored for 20 terminal taxa (Supplementary data) by means of two different, but complementary multivariate and cladistic approaches. These approaches required a reference phylogenetic tree, with realistic branch lengths for the multivariate one, representing the phylogeny of cowbirds and outgroup taxa (Fig. 1A). This molecular scaffold tree was obtained by pruning the maximum likelihood topology of Powell et al. (2014) and using the divergence time estimates from Barker et al. (2015).

In the multivariate approach all characters were analyzed collectively. For the multivariate approach we first calculated a Gower's Coefficient (Gower 1971) matrix derived from the osteological data matrix using the function MorphDistMatrix of the R package Claddis (Lloyd 2016). Then we applied principal coordinates (PCo) analysis to the Gower's matrix to ordinate the data, adding a minimal additive constant to avoid negative eigenvalues as implemented in the cmd scale function of R (R Core Team 2016). The proportion explained by each PCo axis was calculated by the function eigenvals of the R package vegan (Oksanen et al., 2016). With the resultant PCo and the scaffold tree we construct a phylomorphospace in which to explore the occupation of cowbirds and allies using the phylomorphospace function of the R package phtytools (Revell 2012).

The amount and significance of the phylogenetic signal of the skull data was estimated quantitatively on the Gower's matrix by

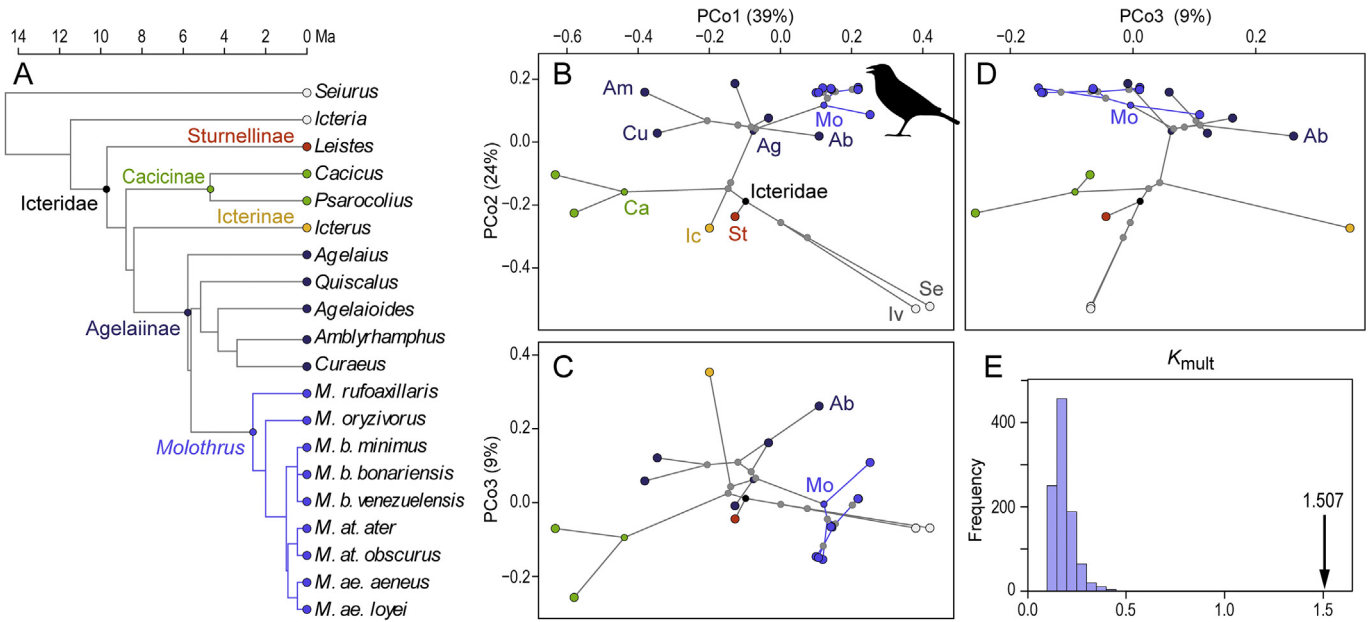


Fig. 1. Phylogenetic signal in the skull of cowbirds and allies. Multivariate approach. (A) Molecular scaffold tree used in the analyses (see section 2.4 for explanation). (B–D) Phylomorphospace constructed by PCoA of Gower's matrix of the skull data. (E) Histogram from the permutation test depicting significance of the K_{mult} value here obtained (arrow). Key to colors of terminal taxa, branches, and nodes of the phylomorphospace are in the scaffold tree. Abbreviations: Ab, *Agelaioides badius*; Ag, Agelaiinae; Am, *Amblyrhampus*; Ca, Cacicinae; Cu, *Curaeus*; Ic, Icterinae; Iv, *Icteria virens*; Ma, Mega-annum; Mo, *Molothrus*; Se, *Seiurus*; St, Sturnellinae (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

using a generalization (K_{mult}) of the K -statistic (Blomberg et al. 2003) for multivariate phenotypes (Adams 2014). Phylogenetic signal was measured by quantifying the amount of variation in the data matrix relative to a randomized sampling of phenotypic values (Adams & Collyer 2019), using the function `physignal` of the R package `geomorph` (Adams & Otárola-Castillo 2013). The significance of the K_{mult} value was then evaluated through a permutation test (1000 iterations) that randomized the data across the tips of the reference phylogeny. This approach was carried out in R v.3.3.0 (R Core Team 2016).

The second approach was cladistic and consisted of Parsimony optimizations of the continuous and discrete skull characters. Since our main goal is testing their phylogenetic signal in the context of the robust evolutionary framework recently provided by molecular-based hypotheses, we conducted a constrained optimization on the abovementioned molecular scaffold tree. However, we also performed unconstrained analyses of the complete data set, as well as of the continuous and discrete character partitions separately, and the most parsimonious trees (MPTs) obtained were topologically compared to the scaffold tree in order to test congruence between different types of data. Topological comparisons were made taking into account the number of shared nodes in the agreement subtree and through the calculation of the weighted subtree-pruning-and-regrafting (SPR) distance between trees, which has been considered a superior measure of topological similarity (Goloboff 2008; Mongiardino Koch et al., 2014). The weighted SPR distance between two trees considers the minimum number of SPR moves required to convert one tree into another, plus the distance (in number of nodes) between two branch positions (Goloboff 2008). Calculations of weighted SPR moves and the derived SPR similarity index (values toward 1 indicate higher similarity), followed the approach of Mongiardino Koch et al. (2014). All these analyses were conducted in TNT v.1.5 (Goloboff et al. 2008; Goloboff & Catalano 2016) and character optimizations were conducted under implied weights. Analyses were performed using different integer values of the concavity constant

($k = 3–20$), but since results were almost invariant we only report on those under a single mild value ($k = 6$). The relative amounts of homoplasy and phylogenetic signal as implied by the scaffold tree were evaluated for the whole data matrix by calculation of overall Retention Index (RI), Homoplasy Index (HI), and Adjusted Homoplasy (AH). To evaluate the relative contribution of individual continuous and discrete traits to overall phylogenetic signal and homoplasy, these same indices (ri , hi , ah) were calculated for each character separately. We also examined the number of transformations between character-states that optimized as unambiguous synapomorphies on each internal branch of the scaffold tree and explored the potential synapomorphies for selected clades.

3. Results

Qualitative inspection of the phylomorphospace constructed upon the PCo of the Gower distance matrix shows cowbirds closely clumping together in a distinct and small region of the skull morphospace, within a domain occupied by Agelaiinae (Fig. 1B–D). Similarly, agelaiines and cacicines also occupy more or less limited areas of the morphospace, separate from other groups, whereas the non-icterid outgroup taxa, namely *Icteria virens* (Linnaeus, 1758) and the parulid *Seiurus Swainson et al., 1827*, lie close to each other distant to icterids (Fig. 1B–D). Among agelaiines, *Curaeus* & *Amblyrhampus Leach, 1814* are sister taxa according to the molecular phylogenies and, consistently, lie close to each other in the skull morphospace (Fig. 1B). *A. badius*, which is now considered to be only distantly related to *Molothrus* cowbirds, lies close to the latter in the morphospace defined by the first two PCo axes (Fig. 1B), but is readily distinguishable in the PCo 3 (Fig. 1C and D). This general pattern in the skull phylomorphospace clearly points to at least some phylogenetic signal in the cranial and mandibular characters here considered.

In our multivariate quantitative estimation of phylogenetic signal we obtained a K_{mult} statistic of >1 , which indicates that the skull of cowbirds and allies carries more phylogenetic signal than

expected under the null hypothesis of random association of phenotypes to the tips of the reference tree. The permutation test finds this value to be statistically significant ($K_{\text{mult}} = 1.507$, $P < 0.005$), with random permutations of the data resulting in K_{mult} values lower than 0.5 (Fig. 1E).

In our cladistic approach, the Parsimony optimization of the cranial and mandibular characters on the scaffold tree yielded index values that also point to presence of phylogenetic signal in the skull data with moderate levels of homoplasy (Fig. 2A–C). Homoplasy values per character (hi, ah) indicate that homoplasy is rather uniformly distributed among continuous characters, more so than among discrete ones, and that both types of characters have similar average scores (Fig. 2A and B). The RI indicates that more than half of the transformations between character-states are retained as synapomorphies across the tree, but character values (ri) show that synapomorphies are, in proportion, mostly from discrete characters (Fig. 2C).

At least one unambiguous synapomorphy optimizes on most internal branches within and outside *Molothrus* (Fig. 2D). Four skull traits emerge as potential synapomorphies of cowbirds (Fig. 2D–F): 1) relatively short premaxilla (2:0.32–0.48; Fig. 2E); 2) moderately long interpalatine process (4:0.59–0.7; Fig. 2F); 3) base of maxillopalatine process exposed in ventral view (16:1; Fig. 2F); 4) quadrate medial condyle round in ventral view (27:1; Fig. 2F). However, none of these character-states is unique to cowbirds and all show at least one instance of homoplasy (the four synapomorphies of *Molothrus* also optimize as apomorphies of *A. badius*), whereas some of them are variable among cowbird species. Notably, most character-state transformations on internal, as well as terminal, branches within *Molothrus* are of continuous characters (Fig. 2D).

Several continuous and discrete skull features also optimize in the nodes corresponding to Icteridae, Cacicinae, Agelaiinae, and minor clades within the latter (Fig. 2D). There are at least 13 traits that emerge as synapomorphic for Icteridae, whereas the distinct

Cacicinae (caciques and oropendolas), which is here poorly represented, is supported by at least ten. Four features (two continuous and two discrete) optimize in Agelaiinae, which comprises cowbirds, grackles, and allies. The internal arrangement of Agelaiinae also appears to be quite supported by skull data, particularly the sister–group relation of *Curaeus* and *Amblyramphus* (Fig. 2D).

The unconstrained optimization of the complete set of characters yields a single fully resolved MPT (Length = 70.267, RI = 0.714, HI = 0.471, AH = 4.286), which shares eight nodes with the scaffold tree, involving 4.045 weighted SPR moves to turn one into another (SPR similarity = 0.762). Analyses of data partitions yield a single, well-resolved MPT both for discrete (Length = 56, RI = 0.742, HI = 0.446, AH = 3.119) and continuous (Length = 12.688, RI = 0.653, HI = 0.513, AH = 0.934) characters. Topological comparisons to the scaffold tree indicate that the tree derived from discrete characters (shared nodes = 9, weighted SPR moves = 2.824, SPR similarity = 0.834) show more congruence to the molecular-based hypothesis than the tree obtained from continuous data (shared nodes = 5, weighted SPR moves = 4.492, SPR similarity = 0.736).

4. Discussion

The molecular ‘revolution’ in avian systematics has provided a robust phylogenetic framework for cowbirds and their kin (Lanyon 1992; Johnson & Lanyon 2000; Powell et al. 2014) and revealed that some traditionally alleged relationships were unsupported by molecular data. This had led some authors to consider morphological data in general as “weakly informative or even—in combination with informal and speculative methods of inference—misleading” (Powell et al. 2014:94–95). Taking into account the early work of Beecher (1951) that lacked a formal methodology or even the more recent study of Björklund (1991), who attempted to reconstruct the phylogeny of grackles based on only a few skeletal characters, this statement may also hold true

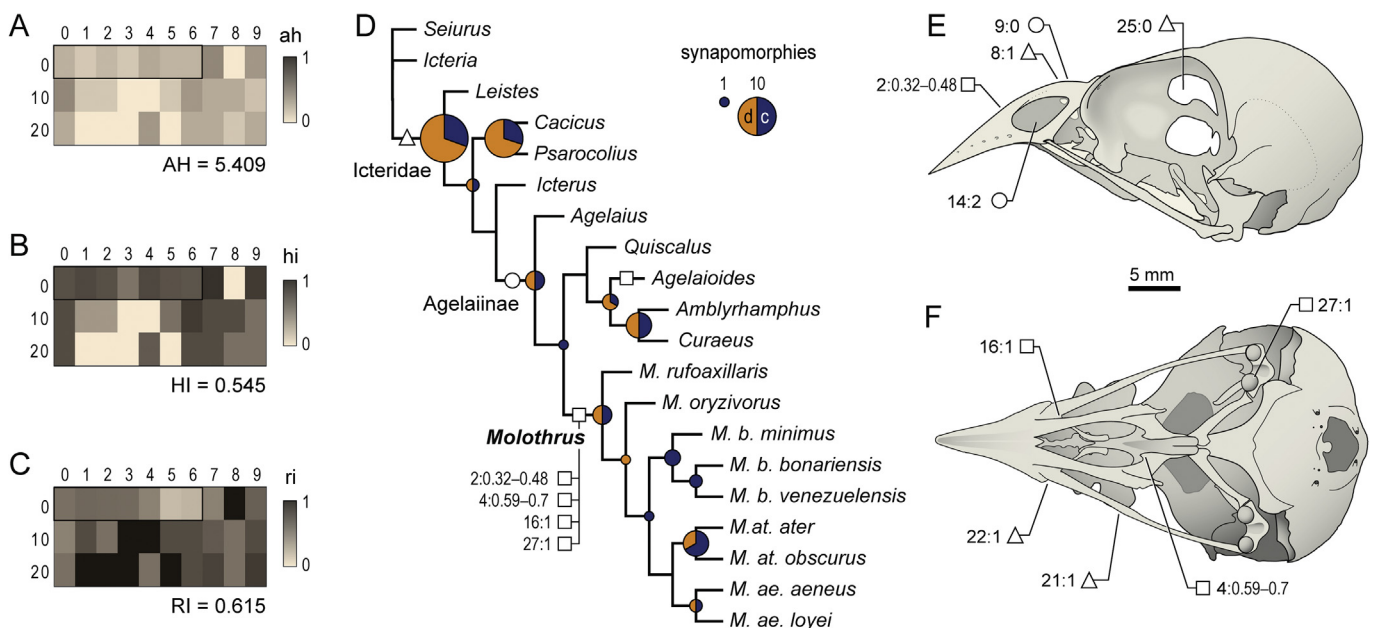


Fig. 2. Phylogenetic signal in the skull of cowbirds and allies. Cladistic approach. (A–C) Phylogenetic signal and homoplasy as indicated by values of (A) Total (AH) and per character (ah) adjusted homoplasy, (B) Total (HI) and per character (hi) homoplasy index, and (C) Total (RI) and per character (ri) retention index. (D) Scaffold tree showing number of synapomorphies and the relative contribution of continuous (c) and discrete (d) characters as pie charts on nodes. (E–F) Schematic drawings of the skull of *Molothrus rufoaxillaris* (MACN 71191) in (E) lateral and (F) ventral views depicting some synapomorphies recovered for Icteridae (triangles), Agelaiinae (circles), and *Molothrus* (squares). The same four synapomorphies of *Molothrus* are also recovered as apomorphies of *Agelaioides* (square). For explanations of characters see Supplementary data.

specifically for skeletal data. However, our results unequivocally indicate that the skull of cowbirds and allies carries phylogenetic signal. This is indicated by the differential occupation of the skull phylomorphospace, with cowbirds occupying a distinct domain close to other agelaiines, which also agrees with the results of the permutation test of the K_{mult} statistic that show that the set of cranial and mandibular traits are significantly more similar among closely related species than if two random species are compared. Moreover, the K_{mult} value here obtained is high when compared with other studies. From an extensive literature survey of over 330 estimates of this statistic from roughly 100 published studies on multivariate phenotypic data of different taxonomic groups, Adams & Collyer (2019) found that most of these obtained K_{mult} values < 1.5 , with a mean phylogenetic signal of 0.65. A high phylogenetic signal has often been interpreted as phylogenetic conservatism (Losos 2008), which, in turn, may be a consequence of quite different evolutionary processes (Kamilar & Cooper 2013). However, disentangling the causes of such phylogenetic conservatism in cowbirds is not possible with current available data.

Our cladistic approach also reveals that the continuous and discrete characters here considered are, to some extent, phylogenetically informative. This is partially reflected in the moderately high overall Retention Index derived from the constrained (RI = 0.615) and unconstrained (RI = 0.714) analyses of the complete set of skull characters, which is high in comparison to that reported for plumage features of grackles and allies (cowbirds included) (RI = 0.11; Eaton 2006), but it is in line to the RI informed for the plumage of orioles (RI = 0.66; Omland & Lanyon 2000) and song characters of oropendolas (RI = 0.88; Price & Lanyon 2002). Likewise, the moderate homoplasy levels in the skull of cowbirds and allies obtained from the constrained (HI = 0.545, AH = 5.409) and unconstrained (HI = 0.471, AH = 4.286) optimizations are higher than those obtained in the latter study (HI = 0.22; Price & Lanyon 2002), but considerably lower than those reported for plumage characters of agelaiines (HI = 0.82; Eaton 2006) and orioles (HI = 0.72; Omland & Lanyon 2000). The only previous cladistic study of skeletal characters of icterids to which comparisons can be made is that of Björklund (1991), who considered 23 discrete (or discretized) characters from different parts of the skeleton (only six were from the skull) of grackles and a few ageline relatives. A reanalysis of his data matrix on a molecular scaffold tree (pruning that of Powell et al. (2014) to match his taxonomic sample) and under our same analytical conditions yields similar overall homoplasy levels (HI = 0.589, AH = 5.050) as those in our skull characters. However, the overall Retention Index for this skeletal data (RI = 0.306) is markedly lower than ours, which is related to the small number of skeletal features recovered as synapomorphies on the scaffold tree.

Conversely, several transformations of the skull characters considered herein optimized on most nodes within *Molothrus*, as well as among outgroup taxa. These constitute potential synapomorphies of cowbirds as a whole, but also for internal relationships within the genus. It has to be noted that the four synapomorphies of *Molothrus* recovered in our analysis are not unique and also optimized as apomorphies of *A. badius* (Fig. 2D), yet this is somewhat expected since baywings were formerly considered as part of *Molothrus* based on their phenotype (Webster 2003). This pattern indicates that extensive homoplasy between baywings and cowbirds already recognized in their plumage and behavior (e.g., Lanyon 1992), also leaks out in skull morphology. Remarkably, the relatively good values of weighted SPR distance obtained from topological comparisons between the MPTs derived from all characters, and particularly from the discrete character partition, and the scaffold tree point to congruence between osteological and molecular data. In this regard, the skull characters here studied also

furnish potential synapomorphies of several clades that are well supported by molecular evidence, but are still poorly diagnosed morphologically. Agelaiinae, the large icterid radiation that includes cowbirds, grackles, and several other blackbirds, is one such clade since it has “no known morphological characters” so far diagnosing it (Remsen et al. 2016:291). Here we recover four features that may contribute to its morphological diagnosis, including one cranial trait related to the ossification pattern of the nasal septum (14:2; Fig. 2E) that does not show homoplasy among sampled taxa. Moreover, several skull characters also appear to support *Curaeus* and *Amblyrhampus* as sister taxa within Agelaiinae (Fig. 2D), a relationship not previously envisaged based on morphological data. However, further analyses with a more extensive taxonomic sampling are warranted to test the actual usefulness of these characters in diagnosing Agelaiinae and minor clades within it.

Our results reveal that cowbirds and their kin show variation in their skulls that, to some degree, is informative of their phylogeny. Moreover, when this kind of data is analyzed with formal quantitative methods under an explicit evolutionary framework, homoplasy can be adequately detected and measured and variation in the some skull characters proves to be useful in diagnosing *Molothrus* as well as other clades that are poorly diagnosed to date. Notably, this integration of skeletal data of cowbirds and allies in the phylogeny admit the incorporation of data from the fossil record (Oswald & Steadman 2011, 2015), which in turn would allow refinement and additional testing of divergence times estimated from molecular data (Barker et al. 2015). This first comparative approach to the skulls of cowbirds and their phylogenetic signal is hence promising and raises the possibility of more integrative studies of their biology and evolution, while encouraging further osteological analyses in cowbirds and other icterids as well.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcz.2020.03.005>.

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