



ECOSYSTEMS

Viability of sex identification of the blue-fronted Amazon parrot (*Amazona aestiva*) based on iris color sexual dichromatism

ANTONIO I.S. NETO, EDMA S. DE ANTONIO, LAIZE TOMAZI, MÁRCIO B. DA SILVA & RICARDO E. FRAGA

Abstract: Reintroducing apprehended wild animal in a natural environment is a complex process that involves many steps, including rehabilitating individuals and ensuring viable populations for reproduction; as such, the proportion between males and females to be reintroduced need to be considered carefully. The need of specialized techniques to identify sex on species that do not present discernible sexual dimorphisms can be a hardship to a successful reintroduction. *Amazona aestiva*, one of the most apprehended species on Brazil, is an example of such case, as sexing techniques employed for it often involves molecular or surgical procedures. Some authors, however, describe potential sexual dimorphisms that could be discernible to the human eye, one of those being an iris color dichromatism between males and females that could present a more conventional way to discern sex on this species. We analyzed the viability of sex identification by direct observation of iris sexual dichromatism, comparing suggestions by professionals familiarized with the species to molecular sexing by Polymerase Chain Reaction (PCR) and measuring color similarity between individuals using digital colorimetry. We found no significant correspondence between sex indication based on direct observation and molecular sexing results, and no relationship between iris color and sex by colorimetric analysis.

Key words: Sexual dichromatism, molecular biology, blue-fronted amazon, colorimetry, wild animals.

INTRODUCTION

The *Amazona* genus is generally known for its parrots with vivid colors and ability to mimic sounds (Toft & Wright 2016). Species from this genus can also present complex social behavior associated with sexual selection, although generally considered to be monomorphic (Schodde et al. 2013). Therefore, techniques to determine the sex of those species often involve surgical procedures such as laparoscopy (Taylor 1994) or specialized techniques such as magnetic resonance tomography (Grando, unpublished data). One of the most

accurate, safe and recommended methods for determining sex on *Amazona* and other avian species is molecular sexing by Polymerase Chain Reaction (PCR). Amplifying genomic DNA with PCR allows the visualization of sexual alleles through electrophoresis, ensuring 99% of accuracy (Griffiths et al. 1998). Molecular sexing is, however, a complex technique that requires specialized equipment and reagents to be applied.

Between the *Amazona* species, *Amazona aestiva* (Linnaeus, 1758) stands out as one of the most common within the illegal bird trade (ICMBio 2017). The high demand for commercialization of

A. aestiva lead to it being considered one of the most apprehended avian species of Brazil (Costa et al. 2018). Institutions responsible for settling, rehabilitating and reintroducing apprehended individuals in a natural environment, such as Wildlife Screening Centers (CETAS), may face adversity reintroducing this high volume of individuals, considering that one key objective for a successful reintroduction is to guarantee a viable composition of males and females in a reintroduced population, and the high cost of sexing techniques available for *A. aestiva* (Armstrong & Seddon 2008, BRASIL 2008, Barros et al. 2017).

However, sex identification of *A. aestiva* could be a less expensive and specialized procedure as some authors suggest evidence of sexual dimorphism presented in some characteristics of *A. aestiva* morphology's, such as body size (Berkunsky et al. 2009) and plumage color (Santos et al. 2006). Sick (1997) describes an apparently sexual dichromatism presented in iris coloration that could be discernible to the human eye; in his description, male individuals would present an orange-yellow iris and females an orange-red one. In avian species, the iris structure evolution is a topic associated to a plethora of hypothesis, with discussions about the functions of iris color patterns ranging from its role in age and sex identification during mate selection (Craig & Hullely 2004, Nogueira & Alves 2009) to its association with activity rhythm (Passarotto et al. 2018); some authors even suggest the possibility of iris color being correlated with light perception and vision accuracy (Savalli 1995).

We analyzed the viability of sex definition of *A. aestiva* by direct observation of this iris sexual dichromatism, comparing sex suggestions by conservation professionals with molecular sexing by PCR and measuring iris color similarity between male and female

individuals using digital colorimetry. An analysis on this matter could benefit field studies as well as conservation practices involving this species, validating a convenient alternative for sex identification or preventing incorrect sex classification based on a subjective definition.

MATERIALS AND METHODS

Data collection

All activities conducted were approved by Federal University of Bahia's Multidisciplinary Health Institute (IMS/UFBA) Ethics Committee on the Use of Animals (CEUA- n 050/2017). Animals analyzed were hosted at Wildlife Screening Center of Vitória da Conquista (CETAS-VDC).

High quality photographs of the animal's eyes were taken using a digital camera (Nikon D3200 with AF-S DX NIKKOR 18-55 mm VR II lenses, using automatic settings for saturation, exposure, white balance and focus) provided by the wildlife screening center. Photographs were taken under indirect natural lightning, maintaining a medium distance of 1 meter between the photographer and the animal, and were saved in JPEG format. Prior photography, animals were captured and handled by professionals at the wildlife screening center. These parameters (especially the distance of photography and absence of external lightning) were taken to minimize startling the animals, as this could lead to a contraction or expansion of their pupils, a circumstance that could impact our analysis.

After photography, biological samples were collected from 44 ringed *A. aestiva* individuals by blood extraction from a cut in the animals' nails. After extraction, blood samples (stored in 110 mm filter paper strips) were transported to the Laboratory of Molecular Biology at Campus Anísio Teixeira, where they were stored in sterilized microcentrifuge tubes and left to dry

for 1 hour in room temperature (20 °C) until DNA extraction.

Sex identification by molecular sexing

DNA extraction was done by washing the paper strips containing blood samples with a buffer solution (Tris 100 mM-0,1% of SDS) and gentle agitation for 30 minutes. After removing the buffer solution, paper strips were washed again with ultrapure water and subjected to gentle agitation for another 30 minutes. After removing the water used for washing, another 50 µL of ultrapure water was added to the tubes and the latter was submitted to a heated bath (90 °C) for 20 minutes. Lastly, the paper strips were discarded and the supernatant containing extracted DNA was stored at -20 °C in a refrigerator until analysis.

Through polymerase chain reaction (PCR) using the enzyme Taq DNA Polymerase and a thermocycler, DNA fragments previously extracted from blood samples were amplified. Sex identification for each individual was provided by visualizing DNA bands after an electrophoretic run. Males were identified by the presence of a single band, representing the Z allele in homozygosis, while females were identified by the presence of two bands, referring to the Z and W alleles. Alleles were judged according to their size, whereas 396 bp characterized the Z allele and 412 bp the W allele (Barros et al. 2017).

After molecular sexing, photographs were identified by sex and order of analysis, with male individuals distinguished by the initial letter "M" and female individuals by the letter "F" (ex: M_01, F_01 and so on).

Sex identification by direct observation

The photographs of *A. aestiva* eyes were presented to three different professionals (two veterinarians and one biologist) with experience

in wildlife conservation and in contact with *A. aestiva* for more than 8 years. Along images, a form was given to each observer for iris color classification in "orange-yellow" or "orange-red", according to the described sexual dichromatism.

Image processing

Prior to colorimetry analysis, photographs were edited using the open-source graphic editor GIMP 2.10.14. Firstly, we used the fuzzy selection tool to select and isolate the region of interest for our analysis, which was defined as between the outer limit and the inner limit of the iris, just before a brown region that demarcates the maximum range of pupil expansion (Figure 1a). Any obstructions (such as shadows) to the selected area were removed. After isolating the region of interest, a green masking (RGB value: 0, 255, 0) was applied to the background of the picture for the sake of creating a precise parameter highlighting the limit of the region of interest to posterior analysis (Figure 1b).

Colorimetric analysis

Quantitative color profiling was done using the package "colordistance" for the R programming language. This package allows quantifying and categorizing all pixels composing an image individually according to their color; classifying them as coordinates in tridimensional color spaces that allows comparison based on similarity (Weller & Westneat 2019). Before analysis, we set the package to ignore all values composing the green background masking.

Distinct observed iris color patterns were compared within the CIE Lab-space, a perceptually uniform color space that reflect the degree of difference between colors perceived by human vision as values classified in dimensions defined as "a (green-red)", "b (blue-yellow)" and "Luminance" channels (Hill et al. 1997). Color pallets were created by measuring

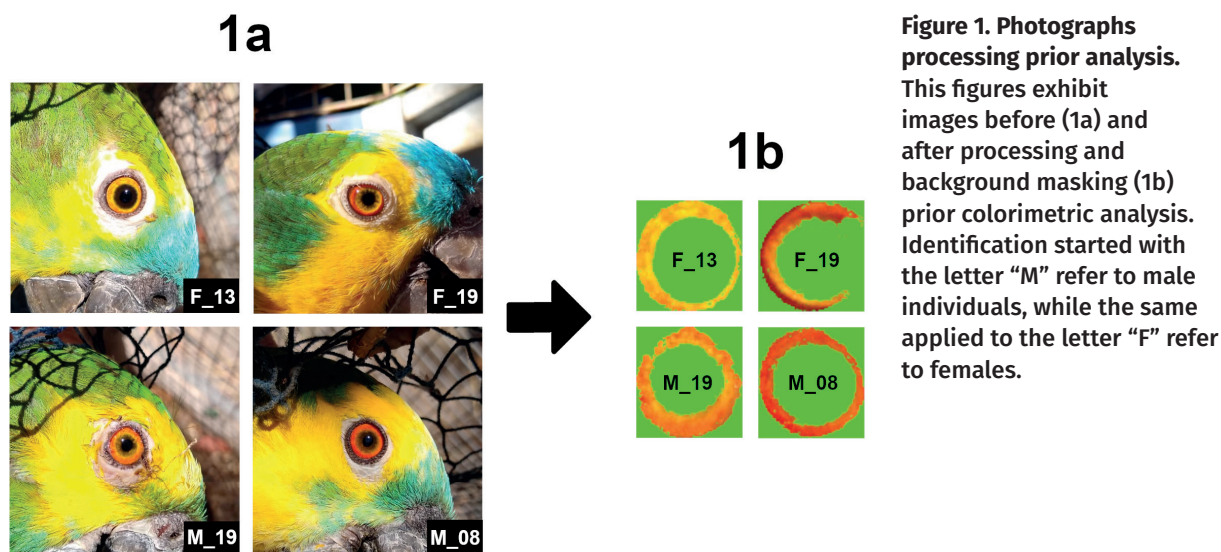


Figure 1. Photographs processing prior analysis. This figures exhibit images before (1a) and after processing and background masking (1b) prior colorimetric analysis. Identification started with the letter “M” refer to male individuals, while the same applied to the letter “F” refer to females.

color composition of each image, emphasizing predominance of similar pixels color values.

Color similarity between males and females' irises were compared within the RGB color space, an additive color model that classifies each pixel in three dimensions according to their distribution of the three primary colors: red, green and blue (Loesdau et al. 2014).

Statistical analysis

To compare sex identification between observers and molecular sexing we used a multiple correspondence analysis (MCA), as well as chi-squared tests comparing accuracy of observers with each other and with PCR results. MCA compares categorical variables based on variance between observations, creating a low-dimensional map that allows visualization of correspondence among variables as points allocated according to similarity between each other (Johs 2018). Color similarity was defined according to RGB value distance between each individual pixel across images using a chi squared metric. Individuals were grouped in a symmetrical distance matrix based on iris color similarity.

RESULTS

We found though molecular sexing by PCR a proportion of 24 male individuals (54.55%) and 20 females (45.45%). Comparing observers' suggestions with molecular sexing (Table 1), our multiple correspondence analysis showed disagreement between sex identification by direct observation of iris dichromatism and molecular sexing by PCR (Figure 2). Accuracy of sex identification based on iris dichromatism was significantly different from accuracy by PCR for each one of the observers ($\chi^2 = 1872.5$, p -value $< 2.2e-16$ compared with a chi squared goodness-of-fit test) although sex suggestions between observers did not differ significantly from each other ($\chi^2 = 0.76688$, p -value = 0.6815 compared with a Pearson's chi squared test).

Associating unanimous color suggestions from observers with quantitative color profiling within the CIE Lab-space, we found no color pattern exclusive to males nor females, with individuals from both sexes presenting orange-yellow and orange-red irises patterns occupying similar ranges within CIE Lab dimensions (Figure 3).

Table I. Observer's suggestions based on iris dichromatism compared to molecular sexing by PCR.

Identification	1° Observer	2° Observer	3° Observer	PCR
F_01	Female	Female	Female	Female
F_02	Male	Female	Male	Female
F_03	Female	Female	Male	Female
F_04	Male	Male	Male	Female
F_05	Female	Female	Female	Female
F_06	Male	Male	Female	Female
F_07	Male	Male	Male	Female
F_08	Female	Male	Male	Female
F_09	Male	Male	Male	Female
F_10	Male	Male	Male	Female
F_11	Male	Female	Male	Female
F_12	Male	Female	Female	Female
F_13	Male	Male	Male	Female
F_14	Male	Male	Male	Female
F_15	Male	Male	Male	Female
F_16	Male	Male	Male	Female
F_17	Male	Male	Male	Female
F_18	Male	Female	Female	Female
F_19	Female	Female	Female	Female
F_20	Female	Female	Female	Female
M_01	Female	Female	Female	Male
M_02	Female	Male	Female	Male
M_03	Female	Female	Female	Male
M_04	Female	Male	Male	Male
M_05	Female	Female	Female	Male
M_06	Male	Male	Male	Male
M_07	Male	Female	Female	Male
M_08	Female	Female	Female	Male
M_09	Female	Female	Female	Male
M_10	Female	Female	Female	Male
M_11	Female	Female	Female	Male
M_12	Female	Female	Female	Male
M_13	Female	Female	Female	Male
M_14	Male	Male	Male	Male
M_15	Male	Male	Male	Male
M_16	Male	Male	Female	Male
M_17	Male	Male	Female	Male
M_18	Female	Female	Female	Male

Table I. Continuation.

M_19	Male	Male	Male	Male
M_20	Male	Male	Female	Male
M_21	Male	Female	Female	Male
M_22	Female	Female	Male	Male
M_23	Male	Male	Male	Male
M_24	Male	Female	Male	Male

Our classification analysis based on color similarity also presented no pattern associating exclusively males nor females. Irises were classified in two main hierarchical groups based on color similarity (Figure 4); one containing 13 individuals (5 males and 8 females) and the other containing 31 individuals (19 males and 12 females).

DISCUSSION

Due to a highly preserved CHD gene presented in sexual chromosomes of avian species, sex identification through molecular markers and electrophoresis visualization is considered one of the most reliable method for sex identification of *Amazona aestiva* and other species from the *Amazona* genus (Griffiths et al. 1998, Ciorpac et al. 2016, Barros et al. 2017). We identified the sex of 44 individuals through molecular sexing, with a proportion of 24 males and 20 females. Prevalence of males is a common recurrence among studies involving molecular sexing of avian species (Nebel et al. 2004, Cerit & Avanus 2007, Barros et al. 2017).

Although sex identification by direct observation of sexual dichromatism can be considered an accurate methodology for some avian species (Farmer & Holmgren 2000), our results imply no significant correspondence between sex suggestions based on iris sexual dichromatism and sex identification by molecular sexing for *A. aestiva*. Color classification, while similar among observers, was not unanimously

defined for all irises observed, indicating a degree of incoherence in distinction between the described iris dichromatism colors, "orange-red" and "orange-yellow", even by professionals familiarized with *A. aestiva*.

Our color similarity matrix also did not present any pattern associating individuals' sex with iris color. Color profiling by software is a developing methodology for phenotypic color pattern characterization (Van Belleghem et al. 2017, Weller & Westneat 2019, Hooper et al. 2020) and has been used before as a method to measure avian species dichromatism (Evans et al. 2010, Cake 2019, Gonzales & Gonzalez 2020). Our converging results between colorimetric analysis and direct observation can attest the validity of this methodology.

Iris color patterns can have a range of functional roles among avian species. In passerine birds, although not common, sexual differences on iris coloration can serve as indicator for both sex and age, with some species presenting a degree of mate selection based on iris color as an indicator to the age of the partner (Craig & Hulley 2004, Nogueira & Alves 2009). There is evidence supporting coevolution of darker iris color patterns and nocturnality in owls' evolutionary histories, with hypothesis associating this correlation as an adaptative feature related to camouflage or concealment (Passarotto et al. 2018). Among Psittiformes, some species of the Cacatuidae family presents discernible iris color sexual dichromatism. This occurs in *Eolophus roseicapilla* (Vieillot, 1817),

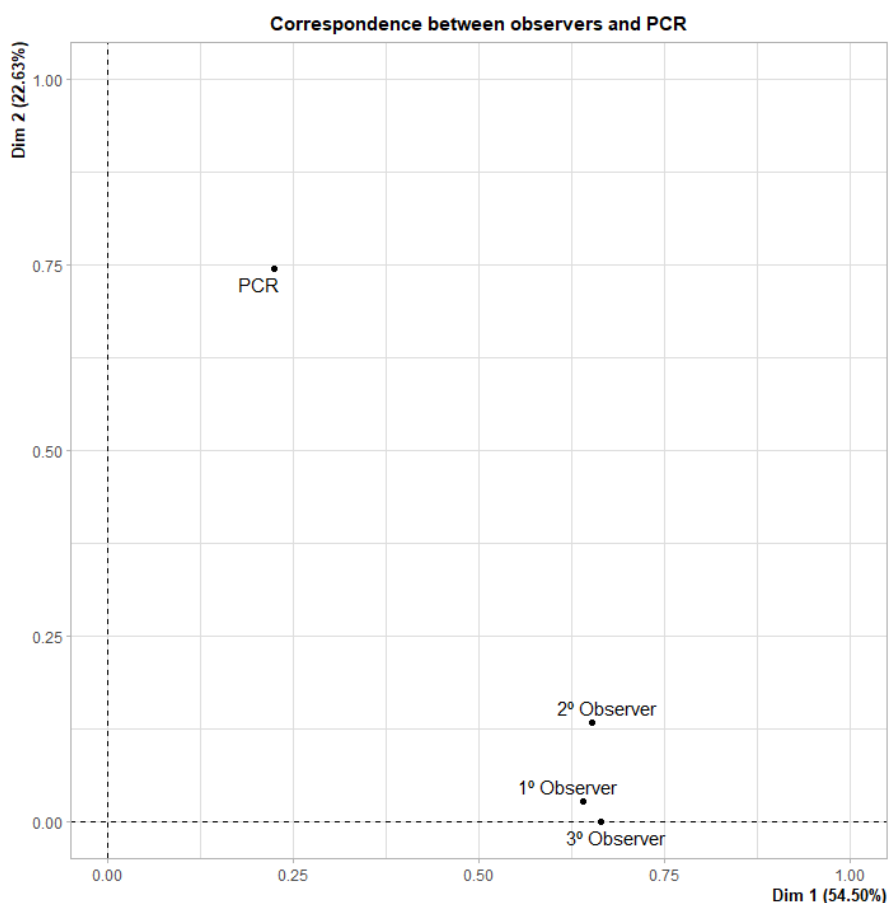


Figure 2. Proximity map relative to correspondence between observers and PCR. This figure illustrates the variables correspondence as a coordinate map, where points are allocated according to similarity or dissimilarity between observations. Variables with a similar profile are allocated closer to each other.

Lophocroa leadbeateri (Vigors, 1831) and *Cacatua Alba* (Müller, 1776), most notably (Forshaw 1989, Cameron 2007). Our results suggest that iris color sexual dichromatism is not present on *Amazona aestiva*, or if it does, it can't be determined by human observation nor digital colorimetry.

It is worth noting that our analysis was conducted with apprehended *A. aestiva* representants. The previously conditions of confinement could have an influence on iris pigmentation, as exposure to external substances has been documented before as a factor associated with changes in iris color patterns among particular bird species (Bortolotti et al. 2003) and captured *A. aestiva* individuals had demonstrated a sensibility to captivity relative to the concentration of androgens and other hormones in bloodstream (Deem et al. 2005). Such components are precursors to carotenoids,

a class of pigments associated with yellow and red iris color patterns in some avian species (Oliphant 1987, Oliphant et al. 1992). A change of concentration of carotenoids related hormones was show to influence pigment deposition related to sexual dimorphism on particular passerine species (Lindsay et al. 2016).

Our results contrast with Santos et al. (2006), who suggests multiple-angle spectrometry as a viable alternative for sexing *A. aestiva* by analyzing plumage color of multiple body regions, such as forehead and wing tip, although this potential sexual dichromatism could not be recognized by the human eye. Berkunsky et al. (2009) suggests morphometrics as a viable alternative for sex identification of *A. aestiva*, as males would present larger size and weight compared to females.

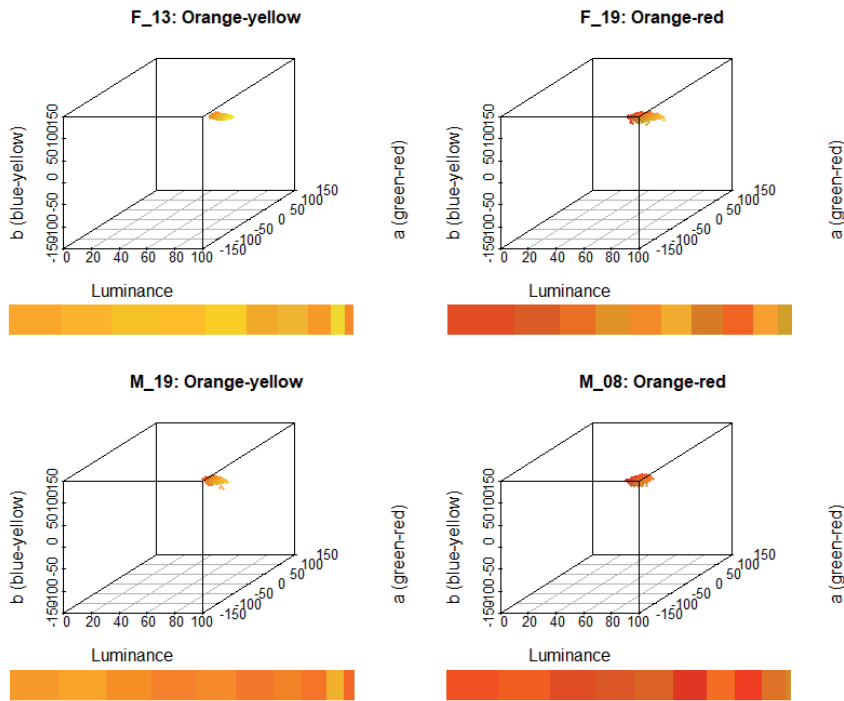


Figure 3. Analysis of color distribution within CIE Lab-space. Color definition for “orange yellow” or “orange red” was defined by agreement of all three observers. The three-dimensional space represents a quantitative classification of color based on Luminance (x axis), green-red (z axis) and blue-yellow (y axis) channels, and each point on the plot represents the color value of each individual pixel composing the image. Below each three-dimensional plot there is a color pallet exhibiting color predominance on each image based on pixel value similarity and distribution.

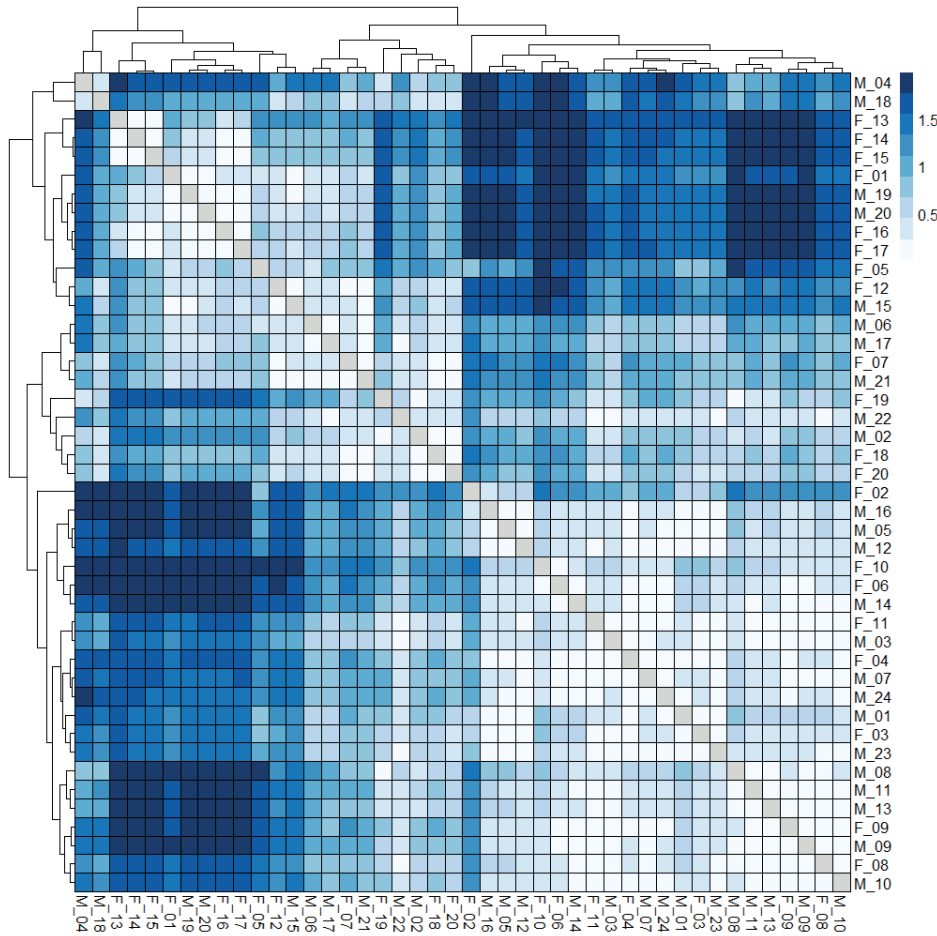


Figure 4. Color similarity clustered heatmap. This figure illustrates iris color similarity between analyzed *A. aestiva* individuals. Identification started with the letter “M” refer to male individuals, while the same applied to the letter “F” refer to females. In this plot, a lower value (lighter color) indicates higher similarity between compared individuals, while a higher value (darker color) designates otherwise. A double dendrogram specify similarity relationships between analyzed individuals, grouping them in clusters based on color proximity.

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Antonio I. Sodrê Neto was responsible for the majority of data collection, data analysis and redaction. Ricardo Evangelista Fraga and Márcio Borba da Silva assisted with data collection including photographs and biological samples assembling. Laize Tomazi contributed with manuscript revision and assisted redaction. Edma Santos de Antônio provided assistance with molecular sexing routines and analysis.

