ORIGINAL ARTICLE

ANATOMICAL LIGHT MICROSCOPIC FEATURES OF THE DOWN FEATHERS OF FOUR SPECIES OF COCKATOOS (AVES: PSITTACIFORMES: PSITTACIDAE)

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ABSTRACT

Four species of commonly traded cockatoos in the Philippines were studied to document and assess the microscopic anatomy of their down feathers and its potential as a method for identification. The samples were analyzed based on node morphology, pigment location, pigment distribution, barbule length, and barbule width. *Cacatua haematuropygia, Cacatua galerita*, and *Probosciger aterrimus* nodes had mixes of expanded, spine, and prong nodes at different degrees throughout, while *Cacatua alba* consistently exhibited prong nodes throughout the pennulum. Pigment distribution in *P. aterrimus* was uniform and internodal and the pigment location ranged from prenodal to nodal, increasing in amount towards the distal end of the barb. The widest barbules were from the male *C. haematuropygia* while the narrowest were from the two female *C. haematuropygia* and the male *C alba*. The mean number of nodes per barbule was highest in one female *C. haematuropygia* while the lowest mean number of nodes per barbule was from *P. aterrimus* Y. Student's T-test and ANOVA test identified potential sexual dimorphism indicators in the mean number of nodes per barbule for *C. galerita* and the mean barbule width for *C. alba* and *C. haematuropygia* and *P. aterrimus*.

Keywords: *Cacatua sp., cockatoo, down feathers, light microscopy, microanatomy*

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INTRODUCTION

The Philippine Cockatoo (*Cacatua haematuropygia* P.L.S. Müller 1776) was categorized by the "Philippine Red List of threatened wild fauna" (2020) as a Critically Endangered species and was categorized by the Convention on International Trade in Endangered Species (CITES) as a one that is threatened with extinction. In a text by BirdLife International (2020), the extremely rapid decline in the bird's population could be attributed primarily to the extensive deforestation in its habitat and continuous poaching due to the high market value that the birds are sold for. Poaching for illegal wildlife trade has also caused the decline of other cockatoo species such as the Umbrella Cockatoo (*C. alba*) and the Black Palm Cockatoo (*P. aterrimus*) and is also becoming a problem for the Sulphur-crested Cockatoo (*C. galerita*) (BirdLife International, 2020; Asian Development Bank, 2019). According to a publication by the ADB (2019), some of the birds are being smuggled into and through the Philippines to meet the demand for caged birds as pets.

In a survey result reported in Tools and Resources to Combat Illegal Wildlife Trade by The World Bank (2018) conducted on experienced wildlife crime enforcement officers with over a decade's worth of operational experience, from seventeen different countries, a large proportion (33%) identified that gaps in species identification tools were integral to the success of impeding wildlife crimes. Wildlife forensics, a method of aiding an investigation of a wildlife crime with

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the use of technology and science, can be used to identify the species involved based on specimens gathered (The World Bank, 2018). One of the methods used in forensic feather identification is light microscopy for identifying specific characteristics in the feathers (Dove and Koch, 2010).

The identification of the morphology of a species' feathers has been used in various fields such as in identifying bird strike encounters by aircrafts, ecology, instances of food contamination, and in law enforcement (Dove and Koch, 2010). Integumentary structures provide external, and therefore more accessible, samples which possess phylogenetic characteristics that remain constant and recognizable, even through external influences and exposures (Chandler, 1916). Morphological convergences may be possible among birds that co-exist in the same habitat or are taxonomically closely similar to each other, such as grebes, loons, and alcids which share similar aquatic environments and feeding behaviors (Dove and Koch, 2010). In identifying feather fragments, it should be possible to identify the avian that it came from up to the lowest possible taxonomic level, and this can best be attained by comparing to reference specimens, the gross characteristics in combination with microscopic characteristics for confirmation (Dove and Koch, 2010).

Currently, information on the light microscopic anatomy of *C. haematuropygia, C. galerita, C. alba,* and *P. aterrimus* are limited. This study aims to provide a descriptive resource specifically for these birds regarding their node morphology, pigment location, pigment distribution, barbule length, and barbule width, to compare these characteristics for differences among these species, and to identify possible demonstrations of sexual dimorphism in the feather microscopic anatomy. Developing a better understanding of the feathers of each bird is hoped to be an advancement in battling illegal wildlife trade, ultimately aiding in the efforts towards species conservation.

MATERIALS AND METHODS

Study Sample Material

The study population consisted of four commonly traded cockatoo species in the country. For the conduct of the sample collections, approval from the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños was obtained (Assigned Protocol Number CVM-2022-013), as well as a Gratuitous Permit (No.: 2018-20 (R3), and a Local Transport Permit (LTP No. PPC-LTP-AO12-2022-687). Ten down feathers, each from one male and one female each of *C. galerita* and *C. alba,* three *P. aterrimus* whose sexes were not determined, and one male *C. haematuropygia* were gathered from the Department of Environment and Natural Resources – Biodiversity Management Bureau (DENR-BMB). Two down feathers, each from two female *C. haematuropygia,* were collected from the Katala Foundation Inc. at the Katala Institute for Ecology and Biodiversity Conservation, Antipuluan, Narra, Palawan (Table 1).

Sample Collection

With the assistance and supervision of the respective handlers of the birds, each bird was caught and properly restrained with their ventral area exposed, avoiding restriction of respiration, and securing the head and limbs. When necessary, a large cloth was used to wrap around larger birds. After a brief examination of the general appearance of the bird, taking photographs for documentation, and observing that it possesses apparently healthy integument, down feather specimens were collected. While wearing clean examination gloves, ten apparently healthy down feather samples were collected from the belly area of the restrained bird, making sure to avoid causing any unnecessary pain and stress. Samples

Table 1. Cockatoo species, sex, and number of individuals.

Species	Sex	Number of birds	Cockatoo Source
Cacatua haematuropygia	M	1	DENR-BMB
Cacatua haematuropygia	F	2	Katala Foundation
Cacatua galerita	M	1	DENR-BMB
Cacatua galerita	F	1	DENR-BMB
Cacatua alba	M	1	DENR-BMB
Cacatua alba	F	1	DENR-BMB
Probosciger aterrimus	Unknown	3	DENR-BMB

from the two female *C. haematuropygia* were limited to two down feathers each in accordance with the restrictions for sampling and transport.The plucked feathers were then kept in properly labelled and sorted paper envelopes. The labelling on the envelopes included the official identification of the bird, the species, whether it was male or female, and the date of collection. After collection, each bird was carefully released into their proper enclosures.

Sample Preparation

Mounting of the specimen onto microscope slides was loosely based on the procedure described by Dove and Koch (2010), and the one described by Flores (2018). The glass microscope slides were labelled with the assigned identification of the birds. Three down barb segments were taken from each of the down feather samples from each of the ten birds. The three barb segments were placed parallel to each other on the labelled glass slide with drops of water and were given designations (I, II, III) from left to right or top to bottom. The water was allowed to evaporate, keeping the barbs in place. These were then mounted with approximately 0.05mL clear nail polish (nitrocellulose + butyl acetate + ethyl acetate), covered with a microscope slide coverslip, then allowed to dry.

Sample Examination

The finished mounted samples were observed under a compound light microscope (Olympus CX43 and CX23, Biological Microscope, Olympus, Tokyo, Japan) equipped with an XCAM1080PHB HDMI+WiFi+SD Card CMOS Camera attachment. Magnification of 100x (LPO) was initially set for observing the patterns of the specimen, and 400x (HPO) magnification was used to observe the node morphology and other identifying characteristics. ToupView® (ToupTek® Photonics, Zhejiang, China), the companion control and processing software to the camera attachment, was used to digitally measure the length, width, and angles, as well as to photo document the samples as viewed through a microscope.

Barbules consist of a base that connect with the rachilla and a pennulum that is made up of many cells taper distally, forming the node (Dove and Koch, 2010). Psittacine barbules lack the villi at the base. Node morphology was noted and described from the proximal, middle, then distal thirds of the barbules located at the middle third of each barb. It was noted whether the node shapes were triangular, ring, crocus, expanded, spine, or prong (Chandler, 1916; Dove and Koch, 2010). When observed under a microscope, triangular nodes appear to have three protruding sides, ring-shaped nodes resemble circular bands along the barbules, crocus nodes have multiple protrusions that resemble florets, expanded nodes are slight protrusions that form a triangular shape, spine have thin, short protrusions, and prong nodes resemble spine nodes except for the tips being longer than the width of the barbule.

For nodes that were identified to be prongs (where the length of the protrusions is greater than or equal to the diameter of the associated internodal section), the prong angles were also measured and noted at the proximal, middle, and distal thirds of each barbule. From these, the mean prong angles per bird were obtained. The mean number of nodes in a representative barbule per bird was also noted.

The presence and absence of pigmentation was observed. Where applicable, it was noted whether the pigment location was located at the nodal, prenodal, or postnodal sections and whether their distribution was uniform (even distribution), internodal (between nodes), or distal (concentrated at the distal portion of the barbule) (Dove and Koch, 2010).

The mean barbule length and mean barbule width were measured in micrometers (µm) based on one (1) barbule located at the middle third of each barb sample. The mean barbule length, mean barbule width, and mean number of nodes per barbule within each species were subjected to a Student's T-test for *C. galerita, C. alba*, and *C. haematuropygia*, and an Analysis of Variance (ANOVA) test for *P. arterrimus* ($p<0.05$).

RESULTS AND DISCUSSION

One of the most diagnostic features that can be observed in cockatoos is the crest that can significantly vary in appearance among the different species (Cameron, 2007). *C. galerita*, for example, exhibits a yellow, forward-curving crest which can change in placement when at rest. The *C. alba* crest is white, backward-curving or lies flat, depending on whether or not it is at rest, while the *P. aterrimus* crest has long, slender, black feathers, which are individually distinct when erect. The crest of some birds may be less prominent, such as the one found in *C. haematuropygia,* which are white, shorter, and more hidden. Plumage colors are then another potential identifying factor, some being the basis of the birds' common names. This can be seen in *C. haematuropygia*, which is also commonly known as the Red-vented cockatoo, and in *P. aterrimus,* which is commonly known as the

Black Palm Cockatoo.

In a publication by Dove and Koch (2010), the microscopic down feather morphology of birds under the Order Psittaciformes was described, though without specific mention of cockatoos. One of the diagnostic features that were common among Psittacine was the lack of villi at the base of cells, which was true for all four of the cockatoo species being studied. The morphology of the barbules appeared to vary along the length of the pennulum.

Examination of the node shapes showed slight progression in the predominance of prong nodes with the narrowest prong angles towards the most distal third of the pennulum for all four species. *C. galerita*, *P. aterrimus*, and *C. haematuropygia* nodes had mixes of expanded, spine, and prong nodes at the proximal third of the pennulum while *C. alba* consistently exhibited prong nodes throughout the pennulum (Table 2). This deviates from the generally expanded nodes that were reported by Dove and Koch (2010) to be the usual node shape for Psittacines. They were described to be greatly expanded throughout the length of the respective barbules.

A study conducted on the selective biodegradation of keratin in feathers strengthened the hypothesis that evolutionary development of rachis was from the merging together of pre-existing barbs and barbules (Lingham-Soliar *et al.,* 2009). This may relate to the difference in node appearances from the base of the barbule to the tip at an evolutionary level. Structurally, the narrowing of prong angles at the most distant end may allow for flexibility of the barb when the barbules interact by maintaining different degrees of interlocking. It was noted by Wilde (2004) that barbules would continue to slide over one another until the nodes meet and become interlocked.

Table 2. Node shapes from each tested *C. haematuropygia, C. galerita, C. alba,* and *Probosciger aterrimus* and the corresponding mean prong angles where applicable.

Species	Node Shape (Mean Prong angle°)		
	Proximal third of pennulum	Middle third of pennulum	Distal third of pennulum
Cacatua haematuropygia			
Male	Expanded, Spine, Prong (21.33°)	Expanded, Spine, Prong (22.77°)	Prong (16.57°)
Female 1	Expanded, Spine, Prong (16.59°)	Expanded, Spine, Prong (24.36°)	Prong (20.13°)
Female 2	Expanded, Spine, Prong (26.25°)	Spine, Prong (20.95°)	Prong (16.35°)
Cacatua galerita			
Male	Expanded, Spine, Prong (24.8°)	Expanded, Spine, Prong (24.11°)	Prong (21.26 °)
Female	Expanded, Spine, Prong (24.74°)	Spine, Prong (23.35 °)	Prong (17.50 °)
Cacatua alba			
Male	Prong (22.97°)	Prong (23.2°)	Prong (19.21°)
Female	Prong (19.82°)	Prong (20.04°)	Prong (14.18°)
Probosciger aterrimus			
X	Spine, Prong (22.45°)	Spine, Prong (22.7°)	Prong (17.56°)
Y	Spine, Prong (19.47°)	Spine, Prong (24.62°)	Prong (17.41°)
Z	Expanded, Spine, Prong (21.96°)	Spine, Prong (20.69°)	Prong (14.78°)

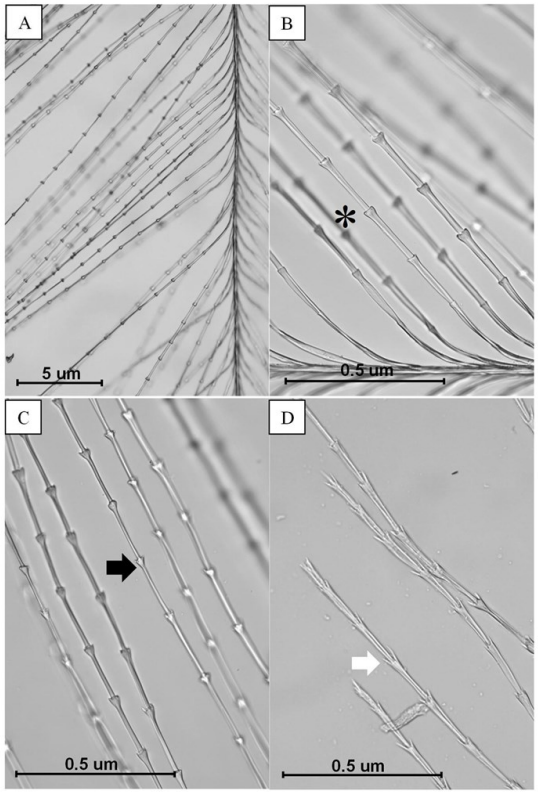

Figure 1. Photomicrographs of barbules from *C. haematuropygia* under 100x magnification (A), and under 400x magnification at the proximal (B), middle (C), and distal (D) thirds of the pennulum. Images show the expanded (asterisk), spine (black arrow), and prong (white arrow) node shapes that were identified.

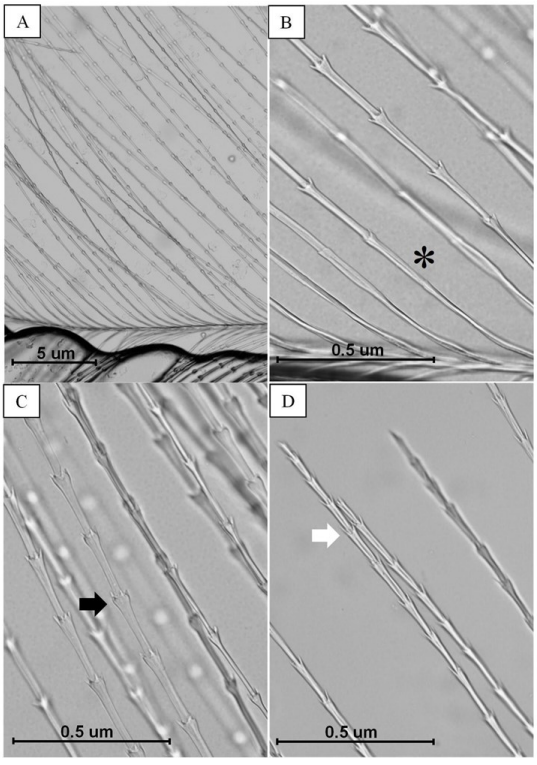

Figure 2. Photomicrographs of barbules from a *Cacatua galerita* under 100x magnification (A), and under 400x magnification at the proximal (B), middle (C), and distal (D) thirds of the pennulum. Images show the expanded (asterisk), spine (black arrow), and prong (white arrow) node shapes that were identified.

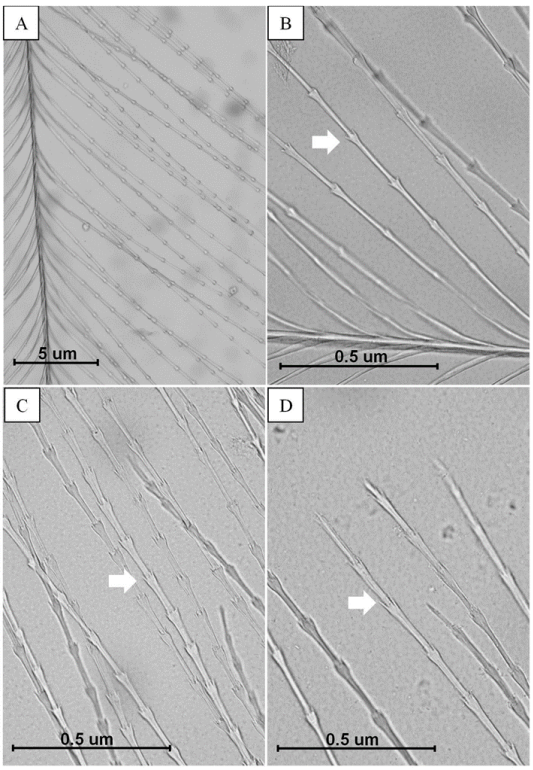

Figure 3. Photomicrographs of barbules from a *Cacatua alba* under 100x magnification (A), and under 400x magnification at the proximal (B), middle (C), and distal (D) thirds of the pennulum. Images show the prong (white arrow) node shape that were identified.

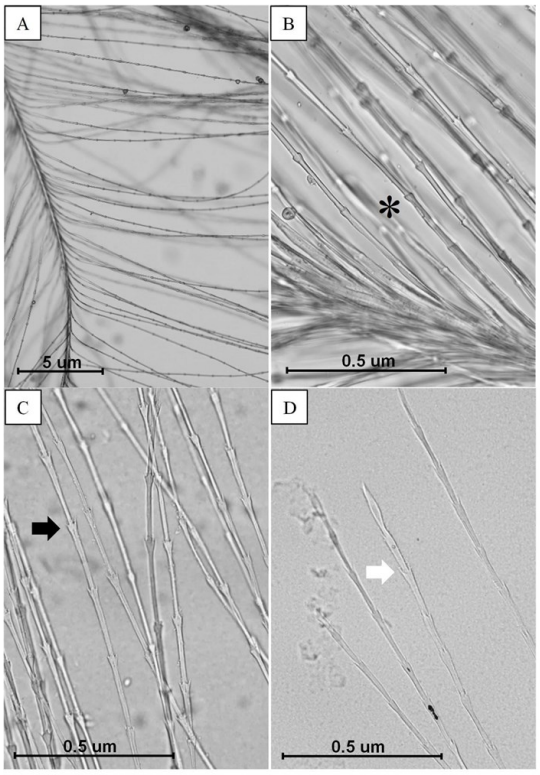

Figure 4. Photomicrographs of barbules from the *P. aterrimus* barbs under 100x magnification (A), and under 400x magnification at the proximal (B), middle (C), and distal (D) thirds of the pennulum. Images show the expanded (asterisk), spine (black arrow), and prong (white arrow) node shapes that were identified.

Gross observation of the obtained down feathers showed significant difference between the *P. aterrimus* feathers and the ones from the other three species.

As *C. haematuropygia, C. galerita*, and *C. alba*, exhibited white down feathers and no pigmented barbules, pigment distribution and location were noted for the three *P. aterrimmus* birds (Figures 6, 7, and 8).

P. aterrimus X and Y had no pigmentations at the proximal third of the barbule while *P. aterrimus* Z had uniform and internodal distribution of pigment for approximately 25% of the proximal third of the barb. Uniform and internodal distribution of pigmentation were more prevalent at the middle third of the barbs, being at about 25% of the section of the barb for P. *aterrimus* X and Y and approximately 75% for *P. aterrimus* Z. For all three birds, uniform and internodal pigment distribution were most obvious at the distal third of the barb (Figure 7). Pigment location, where they were applicable, were varying combinations of prenodal and nodal (Figure 8).

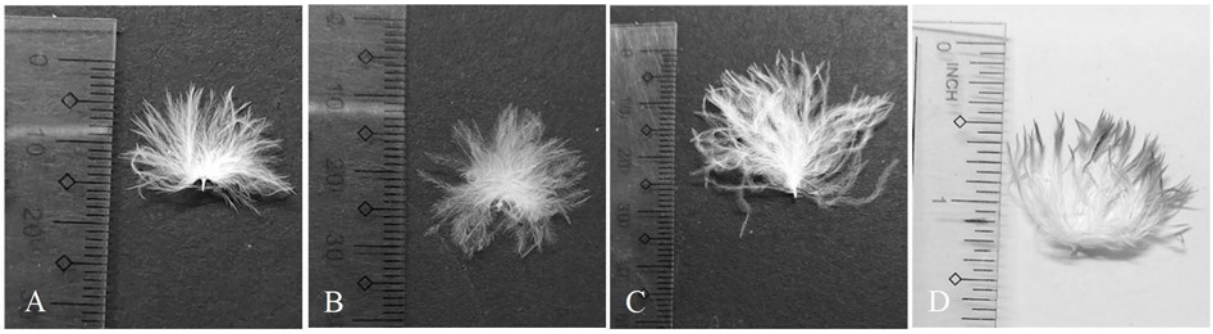

Figure 5. Gross images of representative down feather samples from *Cacatua haematuropygia* (A)*, C. galerita* (B)*, C. alba* (C)*,* and *Probosciger aterrimus* (D). Gradual increase in the dark pigmentation towards the distal ends exhibited in the *P. aterrimus* down feather barbs is observable, in comparison to the three other species.

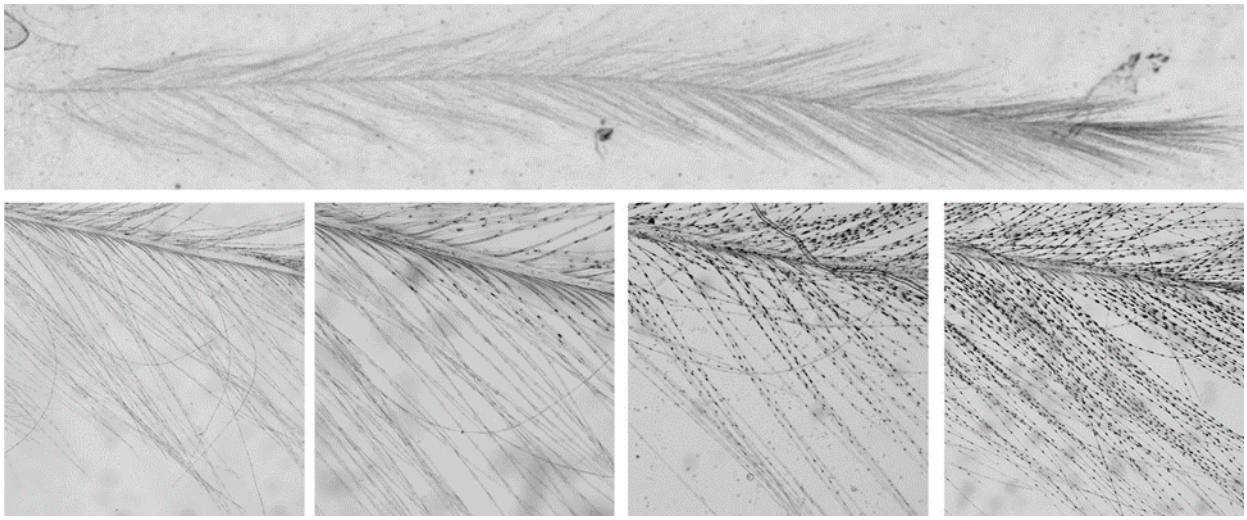

Figure 6. Representation of the pigment location and distribution transition along a *Probosciger aterrimus* barb in 40x magnification (A). Note the lack of pigmentation in the barbules at the base of the barb and the gradual increase in the presence of pigmentation the further away the barbules are from the base at 100x magnification (B, C, D, E).

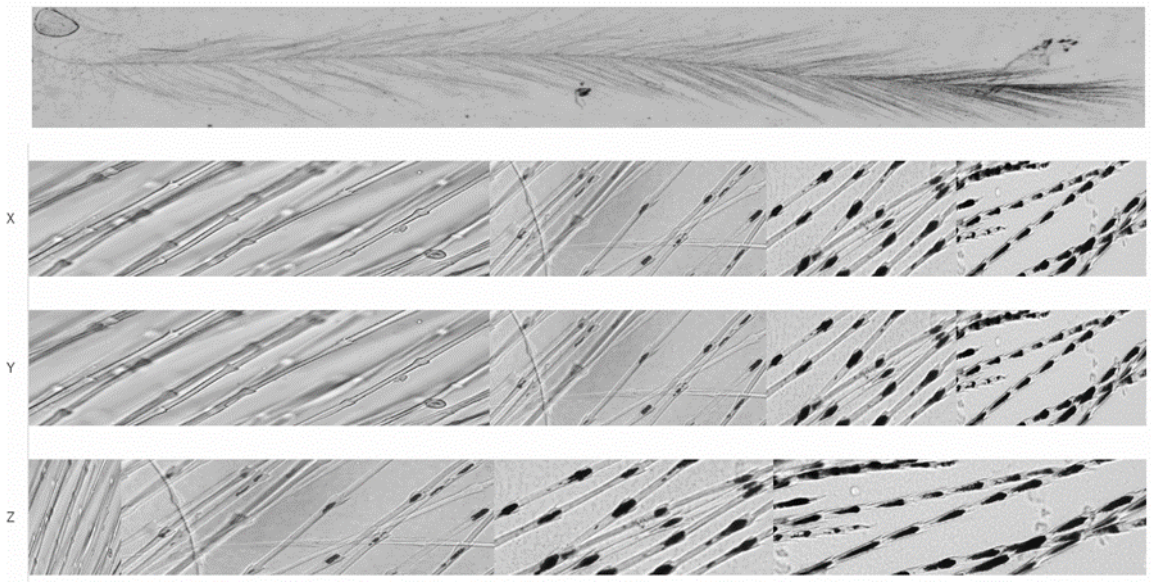

Figure 7. Graphical representation of the pigment distribution identified along the different barbules from *Probosciger aterrimus* individuals (X, Y, and Z) observed compared to an image of a *P. aterrimus* barb to indicate the sections of the images on the barb. The most proximal parts of the barb begin with little to no pigmentation, and gradually reflects the uniform and internodal patterns toward the most distal part of the barb.

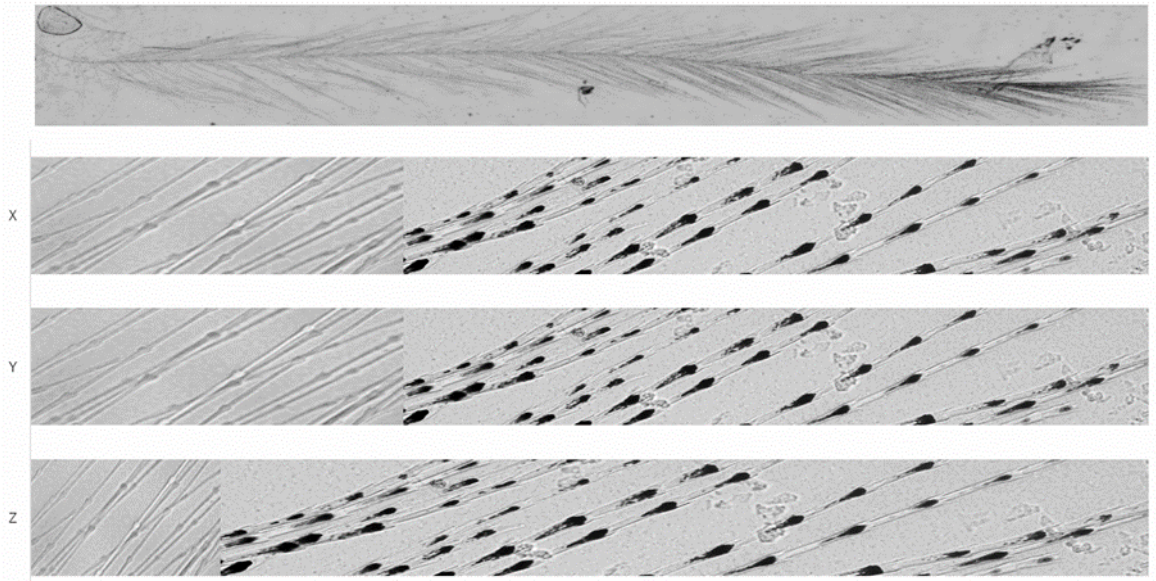

Figure 8. Graphical representation of the pigment locations identified along the different barbules from *Probosciger aterrimus* individuals (X, Y, and Z) observed compared to an image of a *P. aterrimus* barb to indicate the sections of the images on the barb. The most proximal parts of the barb begin with little to no pigmentation, and gradually reflects the prenodal and nodal patterns toward the most distal part of the barb.

Plumage colors convey communication signals which likely evolved to accommodate the visual system of birds (Stoddard and Prum, 2011). Plumage coloration is affected by different pigments such as the species-specific Psittacofulvins in Psittaciforms (Galván, *et al.* 2017). Complex plumage patterns in particular are thought to be produced by melanin, which are endogenously synthesized in specialized cells unlike carotenoid-based pigmentation that are obtained from dietary sources. In a study by Field *et al.* (2013) that investigated a fossilized contour feather with gradient pigmentation with a darker tip and a lighter base as well as the pigmentation gradients in other birds, the pattern is caused by a melanosome concentration gradient. One of their hypotheses for this occurrence is that it serves as an adaptive ecological function, especially since it seemed to occur in numerous distantly related waterbird clades. As melanin is known to cause stiffness in feathers, decreasing amounts of melanin at the base of feathers would allow for decreased stiffness that would be more effective for heat retention. Their second hypothesis was that melanin deposition would require higher energy expenditure and it would therefore be more efficient to restrict pigmentation patterns at the exposed distal portions of the feathers.

Among all of the birds, the mean barbule length was greatest in the *C. haematuropygia* Female 1 at 18.34µm and least in *C. haematuropygia* Female 2 at 14.11µm (Table 3).

The widest barbules were from the male *C. haematuropygia* at an average of 0.42µm, while the narrowest were from the two female *C. haematuropygia* and the male *C. alba* at an average of 0.03µm.

The mean number of nodes per barbule was highest in one female *C. haematuropygia* at 39.83 while the lowest mean number of nodes per barbule was from *P. aterrimus* Y at 33.57.

Table 3. Mean barbule length, barbule width, and number of nodes per barbule of each of the studied *C. haematuropygia, C. galerita, C. alba,* and *P. aterrimus* birds and the significant differences identified between sexes after the first three species were subject to the Student's T-Test and the *P. aterrimus* birds were subject to an Analysis of Variance (ANOVA) test.

Species	Mean barbule length (µm)	Mean barbule width (µm)	Mean number of nodes per barbule
Cacatua haematuropygia			
Male	17.13	0.42***	38.33
Female 1	18.34	0.03***	39.83
Female 2	14.11	0.03***	30.83
Cacatua galerita			
Male	16.93	0.04	37.40*
Female	15.77	0.04	34.17*
Cacatua alba			
Male	14.72	0.03**	35.83
Female	16.11	0.04**	37.17
Probosciger aterrimus			
X	15.13	0.26***	37.27
Y	15.11	0.35***	33.57
Z	14.88	0.32***	35.47

*p=0.013; **p=0.001; ***p=0.000

Subjecting the data to Student's T-test and ANOVA test identified some of the data to be reflective of significant differences. Of the *C. galerita* parameters, a significant difference was found between the mean number of nodes per barbule for the male and female. Analysis for *C. alba* yielded a significant difference between the mean barbule width between the male and female. Similarly, *C. haematuropygia* analysis yielded a significant difference for the male and Female 2 when it came to mean barbule length and mean number of nodes per barbule, and a significant difference between the male and both female birds when it came to the mean barbule width. The ANOVA test for *P. aterrimus* identified the mean barbule width to have a significant difference among the three individuals. The significant differences in the measurable parameters of individuals within the species examined indicate the potential for sexual dimorphism.

In conclusion, identifying characteristics were observed and documented among the four cockatoo species, addressing the limited published work on down feather morphology of cockatoo species in the Philippines. Observation of down feather samples with the use of light microscopy was identified to be a relatively simple and efficient method of narrowing down the potential species of origin. Statistical comparisons of characteristics such as the mean number of nodes per barbule and the mean barbule width also suggest the potential for sexual dimorphism. In combination with other factors and methods of observation, microscopic observation, and comparison with reference documentation may be useful tools in forensic investigations.

It is recommended that further study of down morphology in these birds be conducted by examining samples that are taken from different regions of the body of the bird. Although down feathers are primarily most abundant at the chest and belly areas, the other types of feathers possess downy feathers which might also be worth examining. Additional statistical analysis may also be done to compare the characteristics between species. Identifying significant differences in the values obtained from each species may objectively establish distinguishing characteristics. Comparisons of features between species with similar appearances such as the Sulphur-crested cockatoo (*Cacatua galerita*) and the Yellow-crested cockatoo (*Cacatua sulphurea*) may also be beneficial. Lastly, it is recommended to explore the effects of different mounting mediums on the quality of the mounted samples. Flo-texx® (Lerner Laboratories, Pittsburgh, PA USA) is recommended for samples that will be stored for long periods of time as it does not become yellowish over the years (Dove and Koch, 2010).

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STATEMENT ON COMPETING INTEREST

The authors have no competing interest to declare.

AUTHOR'S CONTRIBUTION

MGP, EALT, and MSF worked on conceptualization, Formal Analysis, Resources Gathering, andManuscript Writing of the project. Data Curation and Investigation by MGP and EALT. FundingAcquisition and Project Administration by MGP. Methodology by MGP and MSF.

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