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Description of the exoskeleton microstructure of *Phanaeus vindex* MacLeay, 1819 (Coleoptera: Scarabaeidae: Scarabaeinae)

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Abstract. Phanaeini is a New World tribe of scarab beetles (Coleoptera: Scarabaeidae: Scarabaeinae) whose members are noteworthy for their iridescence—the phenomenon that a color changes as the angle at which it is viewed changes. Iridescence has evolved independently in various clades of animals, including multiple clades of arthropods. Recently, a team of researchers have described the microstructure of the elytra (the hardened forewing) of the iridescent beetle *Chrysina gloriosa* (LeConte, 1854) (Scarabaeidae: Rutelinae). The North American *Phanaeus vindex* MacLeay, 1819 also displays iridescence; however, the microstructure of *P. vindex*'s elytra has not yet been elucidated. The null hypothesis is therefore that the elytra of *P. vindex* is the same microstructurally as that of *C. gloriosa*. Following analysis, we have accepted the null hypothesis: *P. vindex* displays elytral hexagonal cells called "conic focal domains" that, when viewed from above, are microstructurally similar to those of *C. gloriosa*. The subfamily that is sister to Rutelinae, Dynastinae, is non-iridescent and also lacks domains in its elytra, giving further evidence to the hypothesis that the domains are responsible for iridescence.

Keywords: phanaeine, iridescence, beetles.

Phanaeini (Scarabaeidae: Scarabaeinae) is a tribe of New World dung beetles, being one of three clades of Scarabaeidae beetles with a circularly polarized, iridescent exocuticle (Seago et al. 2009). Three species of *Phanaeus* are present in the southern and eastern US: *Phanaeus vindex* Macleay, 1819, *Phanaeus igneus* Macleay, 1819, and *Phanaeus difformis* Leconte, 1847 (Price 2008). The three species are identifiable in part based on their colors (Edmonds 1994). For *P. vindex*, the posterior part of the head and sides of the pronotum (or prothorax) are golden to yellow-green, the disk of the pronotum is coppery red, and the elytra (hardened forewings) are golden green to dark green.

Some scarab beetles have had their (wax-covered) exocuticle detailed, such as *Chrysina gloriosa* (LeConte, 1854) (Scarabaeidae: Rutelinae), and in this case, it was discovered that the elytra are covered with predominantly hexagonal cells called "conic focal domains" (Sharma et al. 2009). The researchers fluorescently stained the domains, finding that different regions of them were stained differently which indicated different fluorophore orientations. The staining in addition to freeze-fracture sectioning has revealed that domains are formed by concentric layers of chitin and have a convex conical protrusion in the center. Further, the optical properties of their cuticular chitin microfibril layers have been compared with cholesteric liquid crystals (ChLC).

Liquid crystals are defined as "compounds showing a liquid state with optical and dielectric anisotropies between those of a solid crystal and an isotropic liquid which also orient themselves into helically stacked layers" (Tamaoki 2001). There are three ways the stacked layers can be aligned: nematic, in which the molecules' long axes are parallel to each other, but not all aligned in the same plane; cholesteric, in which the axes are directionally rotated; and smectic, in which the molecules are both parallel to each other and aligned within the same plane. In *C. gloriosa*, the cholesteric alignment is most relevant. Any helical structure has a pitch (or period), which is the height of one complete helix turn, and if the pitch falls within the visible light wavelength spectrum, then visible iridescent light is reflected back.

At the interface between the ChLC and the air (the free surface), ChLC form hexagonal regions that are conical in the center (Meister et al. 1996). These hexagonal regions are formed by concentric layers of chitin just like the domains of *C. gloriosa*'s elytra (Sharma et al. 2009). Sharma *et al.* also advanced phenomenological arguments about the domains, relating their optical properties to those of ChLC. In a bright-field microscope, a wide open aperture shows a larger yellow area inside the hexagonal cells, but as the aperture is closed, letting in less white incident light, the yellow area shrinks. To understand this, the researchers have utilized the modified Bragg equation:

$$\lambda = np(\cos(\phi - \theta))$$

Where λ is the wavelength, n is the refractive index, p is the pitch, ϕ is the angle of oblique incident light, and θ is the angle of orientation of the helix with respect to normal surface. Therefore, as the aperture opens, the range of incident light angles increases, and the amount of yellow increases, implying that ϕ-θ is decreasing. As ϕ-θ decreases, cos(ϕ-θ) increases, increasing the wavelength. This is why more yellow appears, since yellow light has a longer wavelength than green light. And as the aperture closes, there is less yellow area, implying that ϕ-θ is increasing. Increasing ϕ-θ therefore decreases cos(ϕ-θ), meaning we should expect greener. In dark field microscopy, only very large incident

Table 1. lists all the beetles used in this study.

Species	Subfamily	Tribe	Iridescence	Distributer	Source	Number of Specimens
Phanaeus vindex MacLeay, 1819	Scarabaeinae	Phanaeini	Present	Real River Supply	Texas	1
Chrysina gloriosa (LeConte, 1854)	Rutelinae	Rutelini	Present	BicBugs	Green Valley, Arizona	1
Eupatorus gracilicornis Arrow, 1908	Dynastinae	Dynastini	Absent	Easier Group USA	Thailand	1

angles are possible, so ϕ-θ is very large, meaning cos(ϕ-θ) is very small. Under dark fields, the yellow center should disappear.

Therefore, the questions are: do the iridescent beetles of Phanaeini have conic focal domains like *C. gloriosa*. If Phanaeini do have them, are they similar morphologically and optically to the domains of *C. gloriosa*? Rutelini and Phanaeini do not form a monophyletic lineage, so if they do possess similar domains, then this would imply convergent evolution in these lineages (*Guo et al. 2022). The non-iridescent subfamily most closely related to Rutelinae is Dynastinae (*Eupatorus gracilicornis* Arrow, 1908 is used to represent this clade) (Tarasov & Dimitrov 2016). Does this subfamily possess conic focal domains?

Light Microscopy and SEM. Microscope images of the elytra of *P. vindex* and *E. gracilicornis* were photographed and characterized. These were carried out under an Olympus BX60 light microscope, a Nikon TS2 light microscope, a Hitachi TM-1000 scanning electron microscope, and an Olympus BX60 light microscope; the Hitachi TM-1000 has the advantage to require no critical point dehydration and no sputtering. Before putting the specimens in the SEM, they were washed with acetone to remove the wax layer.

Microtome: Fixation and Dehydration Series. In preparation for the microtome, first, the specimen was fixed in a modified Kahle's solution (Tab. 2), evacuated in a vacuum device for five to fifteen minutes, and then was stored in 75% ethanol for more than 24 hours.

The specimen was dehydrated through a graded alcohol series (Beutel et al. 2014; Barbosa et al. 2014).

1. Specimen was placed in 75% ethanol for an hour and then step 1 was performed again.
2. Specimen was placed in 85% ethanol for an hour.
3. Specimen was placed in 95% ethanol for an hour.
4. Specimen was placed in 100% ethanol for an hour.
5. Specimen was placed in a second aliquot of 100% ethanol for over a day.

Table 2. Components of Kahle's Modified Solution

Insect Fixative	Kahle's Modified
Formaldehyde conc.	Formalin 15 parts
Ethanol 95%	16 parts
Glacial acetic acid	1 part
H_2O	19 parts

Microtome: Paraffin Embedding. Following dehydration, the specimen was prepared for paraffin wax immersion.

1. FisherBrand Paraplast wax was melted to keep a sufficient supply at 60 °C.
2. Specimen was immersed in a solution of 2:1 ethanol and xylene for at least an hour.
3. Specimen was moved to a solution of 1:1 ethanol and xylene for at least an hour.
4. Specimen was moved to a solution of 1:2 ethanol and xylene for at least an hour.
5. Then, the specimen was moved to a pure xylene solution (specimen can also be stored in xylene solution) for at least an hour.
6. Specimen was embedded in a solution of 1:1 xylene and molten Paraplast for at least an hour.
7. Specimen was immersed in molten pure Paraplast overnight.
8. The paraplast was replaced to reduce the xylene content and Step 7 was repeated, letting the residual xylene evaporate. The sufficient elimination of the xylene was tested by scent.

Microtome: Wax block embedding. Next were the wax block embedding steps.

1. Glycerin was used to line the mold into which the specimen was put.
2. Paraffin and specimen were quickly poured in to the mold. A pre-heated pair of forceps was used to orient and adjust the specimen in the liquid wax.
3. Once the surface of the wax became opaque white from solidifying, the mold was moved to an ice water bath. The wax would contract which on one hand could cause weakness in the paraffin block, but also helped releasing the block from the mold. Because the specimen is dark, the specimen was visible through the paraffin inside of the paraffin ingot.
4. The released wax ingot was then trimmed around the specimen to fit of the section intended and welded onto a wooden block for the microtome.

Microtome Sectioning and Staining. Initially, the specimen was cut on a Thermo Scientific Shandon Finesse 325 rotary microtome at 10 µm and mouted on object carrier slides with a lysine coating as adhesive.

Differential staining was with a simplified Safranin/Fastgreen according to Ruzin (1999), attempting a differentiation of protein components from carbohydrate-like chitin, leaving aliphatic waxes unstained.

When the elytra was sectioned by the microtome, the elytra was found to be too tough for the blade to cut. Instead, each time the blade went through the elytra, the elytra was broken by the blade. Thus, another elytra was prepared exactly the same as before but was put through a softening procedure before cutting. This softening procedure included the following steps (Barbosa et al. 2014):

1. Specimen was put into 10% potassium hydroxide solution for 24 hours.
2. Specimen was washed in water for 6 hours.
3. Specimen was transferred to a 33% acetic acid solution for 24 hours.
4. Finally, specimen was washed in water for 6-8 hours.
5. All other fixation, dehydration, and wax-embedding steps were the same.

Cryo-Microtome Embedding and Sectioning. However, despite applying the softening procedure, the elytra was still not amenable to cutting. At this point, LSU Health Shreveport was contacted.

1. The *P. vindex* elytra were embedded in PolarStat Plus Embedding Medium.
2. Following embedding, the elytra were mounted on the Leica CM3050S cryo-microtome.
3. 10 µm sections were cut from both species.

No iridescence was observed when light was shone from underneath the elytra using the light microscope, indicating that iridescence results from the dorsal exocuticle of the elytra. Further, whereas *C. gloriosa*'s elytra is quite smooth overall, the epicuticle of *P. vindex* is highly irregular. *P. vindex* does indeed possess conic focal domains in its elytra as shown by the SEM (Figs. 1 and 2). The elytral cells in *P. vindex* are distributed in a similar manner to *C. gloriosa*. Like in *C. gloriosa*, the cells of *P. vindex* are about 10µm in width. In some areas of the elytra, the domains are immediately visible under the microscope; however, in other areas, the domains are covered (Fig. 2).

When observing the domains, we conjectured that the domains were exhibiting a lens effect. In essence, light was passed through a clear layer and focused onto the domains. When the elytra of *P. vindex* was sectioned by the cryo-microtome, this revealed the layers of chitin (Fig. 3). The top layer, the epicuticle, is clear, and beneath the epicuticle is the dark exocuticle where the domains are.

When the non-iridescent *E. gracilicornis* was placed under the light microscope, the elytra were observed to completely lack the domains (Fig. 4). This absence of domains in conjunction with non-iridescence is evidence in favor of the hypothesis that the domains cause iridescence.

There are striking similarities in the iridescent mechanism of *P. vindex* and *C. gloriosa*. To answer the first question posed (whether *P. vindex* has conic focal domains like *C. gloriosa*), both species do indeed possess the domains. The domains are the same size in both species as well, being approximately 10 µm. The existence of hexagonal domains in *P. vindex*'s elytra and the presence of iridescence imply that the chitin layers within the elytra are helically stacked in accordance with the known properties of cholesteric liquid crystals.

The second question (whether the domains are similar morphologically underneath and optically) is currently partly unanswered due to technological limitations. Even after applying a softening solution to the elytra, it was still not amenable to cutting by our rotary microtome. LSUS also does not have a transmission electron microscope that could be used to investigate the chitin layers of *P.*

vindex's elytra, and the closest available TEM is currently unavailable due to financial concerns. By sectioning the elytra with the cryo-microtome, we did find that the elytra exhibit a lens effect. Light must pass through the clear epicuticle and be focused on the chitin layers in the exocuticle. Further, we were unable to determine if *P. vindex*'s domains act optically similar possibly due to not having the same light microscope as Sharma et al. (2009).

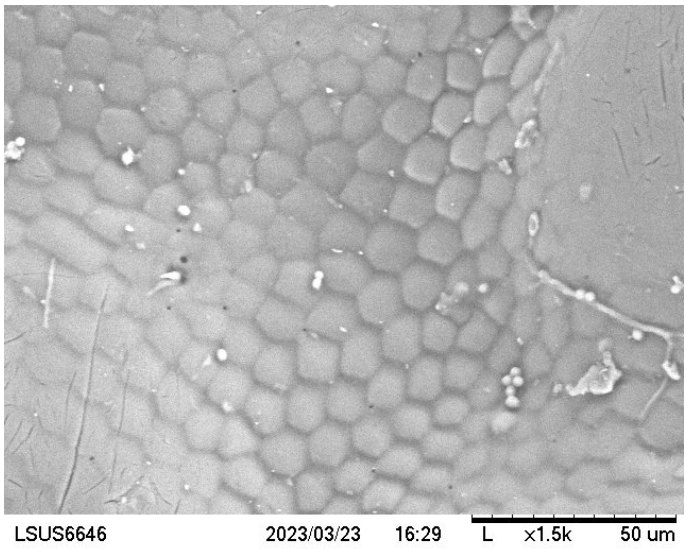

Figure 1. *Phanaeus vindex* MacLeay, 1819 (Coleoptera: Scarabaeidae: Scarabaeinae) elytra. 1500x magnification.

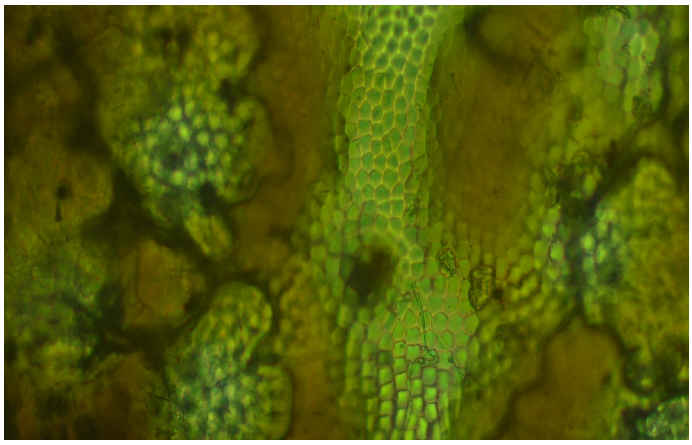

Figure 2. *Phanaeus vindex* MacLeay, 1819 (Coleoptera: Scarabaeidae: Scarabaeinae) elytra. Elytra is curved, so only a portion of it is in focus.

The third and fourth questions (whether Eucraniini and Dynastinae have the domains in their elytra) has been partially answered as indicated by Tab. 3. The fact that *P. vindex* and *C. gloriosa* have the domains but Dynastinae does not (Fig. 4) implies at least two separate instances of convergent evolution. We were unable to obtain members of Eucraniini in the requisite time frame, so this question is currently unanswered.

Table 3. Beetles Compared with Iridescence and Conic Focal Domains

Species	Iridescence	Conic Focal Domains
Phanaeus vindex MacLeay, 1819	Present	Present
Chrysina gloriosa (LeConte, 1854)	Present	Present
Eupatorus gracilicornis Arrow, 1908	Absent	Absent

The evolution of the underlying structural mechanism that gives rise to multilayer reflecting seems to be a byproduct of the multilayer cuticle that beetles already have (Seago et al. 2009). Likely for this reason, multilayer reflectors have repeatedly evolved in beetles. Simple changes in the thickness of the chitin or melanin layers could generate a variety of colors. Further, the chitin layers are already helically deposited in beetle larvae, but the layers become deposited in parallel fashion in adults (Seago et al. 2009).

Thus, neoteny (the retention of offspring traits into adulthood) could explain the origin of the domains in Phanaeini and Rutelinae.

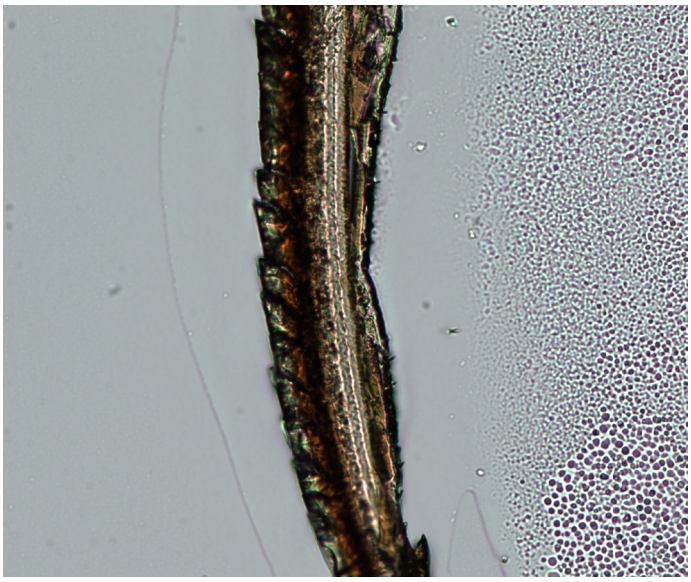

Figure 3. *Phanaeus vindex* MacLeay, 1819 (Coleoptera: Scarabaeidae: Scarabaeinae) elytra. 200x magnification.

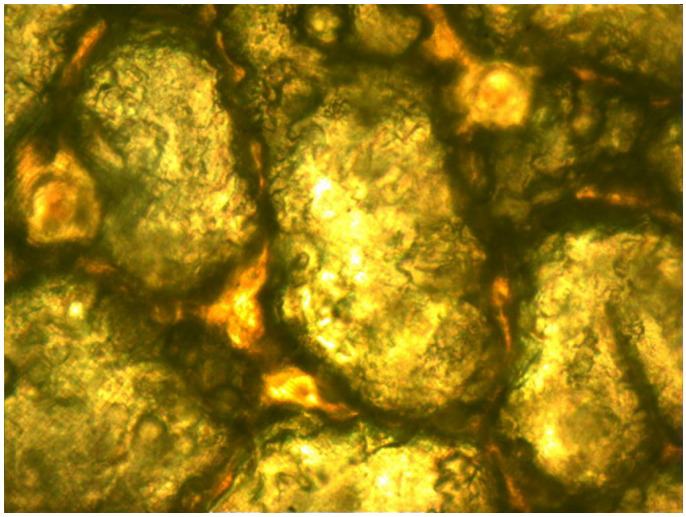

Figure 4. *Eupatorus gracilicornis* Arrow, 1908 (Coleoptera: Scarabaeidae: Dynastinae) elytra. 100x magnification.

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Authors' Contribution

JW: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; MB: Conceptualization, Methodology, Project Administration, Resources, Supervision, Validation, Writing – review & editing.

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Only organisms used were already deceased.

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All data is available in manuscript.

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