

Microstructural investigations of wing scales of three *Cupido* Schrank (Lepidoptera: Lycaenidae: Polyommatainae) species

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Abstract. Microstructural analyses in plant and animal taxa have revealed interesting features, implying ecological rationale. In lycaenids, scales of the wings possess several types of microstructures which, by layout and composition, assign unique properties to the scales. Due to different optical attributes of scales of distinct species, variability of mentioned structures was found. Considering that evolutionary factors have generated such variability, it could be worthwhile to see if quantitative parameters of scale structures are specific to different taxa. The current paper investigates such parameters in three species of the *Cupido* genus, *C. argiades* (Pallas, 1771), *C. decoloratus* (Staudinger, 1886) and *C. alcetas* (Hoffmannsegg, 1804), for which quantitative data is missing in literature. Distances between ridges and ribs, number and area of holes and filling factors were calculated from scanning electron microscope images. Statistical differences were found for some of the parameters between the analysed species for scales with structural colour properties. The findings illustrate a variability that adds to other similar studies reporting microstructural variations on lycaenid taxa.

Keywords: lycaenids, ridges, ribs, holes, interspecific differences.

Introduction

One of the most obvious traits of many lycaenids but also of species from other butterfly families is the vivid, often iridescent colours. This feature is the result of the evolutions of this group of organisms in the course of millions of years and possibly involved in sexual communications as well as in cryptic behaviour (Silberglied & Taylor 1978, Rutowski 1981, Krebs & West 1988, Fordyce et al. 2002, Biró et al. 2008, Vukusic & Stavenga 2009, White et al. 2012). Researches of the XXth century (Onslow 1921 cf. Tilley & Eliot 2002, Mason 1926, 1927 cf. Tilley & Eliot 2002) revealed that butterfly colours are generated through two distinct mechanisms, one involving pigments (chemical colours), the other relying on physical characteristics of wing scales (structural colours), more precisely on microscale structures of the scales (Vértesy et al. 2006). Such scales, when lacking pigments, are colourless and considered transparent to light (Tilley & Eliot 2002, Kertész et al. 2006).

Scales with structural colours are composed of a lower, weakly structured layer and an upper layer with numerous structures in the shape of longitudinal ridges connected by transversal ribs (Downey and Allyn 1975, Ghiradella 1991, Ghiradella 1998 cf. Giraldo 2007, Ghiradella & Butler 2009). These structures are chitinous, while the

spaces between them consist of air, thus the scale is composed of substances with different light refraction indices. The scale is therefore reflecting the light in different ways, according to the disposition of microstructures (Biró et al. 2008, Ingram & Parker 2008, Saranathan et al. 2010, Tamáska et al. 2012). Considering the spatial periodicity of the various microstructures, they are regarded as photonic crystals, thin film reflectors or diffraction gratings which reflect some light wavelengths while blocking others (Kertész et al., 2006, Vértesy et al., 2006).

The shapes and patterns of scales microstructures are variable, both at infra- and suprageneric levels (Bálint et al. 2004, Vértesy et al. 2006, Mika et al. 2012). The structures can be presented as ridges, ribs, lamellae or perforated layers and, due to light manipulation capacities, they play an important role in intra- and interspecific relations. The vivid colours and UV related properties generated are most possibly involved in male-male interactions in the case of conspecific individuals, during territorial behaviours (Rutowski 1977, Lederhouse & Scriber 1996, Kertész et al. 2006, Bálint et al. 2007). Moreover, these colours are used by males to select females (Knuettel & Fiedler 2001) and by females to recognize conspecific males and for further mating selection (Breuker & Brakefield 2002, Robertson & Manteiro 2005, Kertész et al. 2006, Friberg et al. 2008, Tamáska et

al. 2012). The involvement of photonic crystal type structures in visual signalling is suggested by the finding that different species possess different structures which generate distinct colours or hues (Tilley & Eliot 2002, Vértsey et al. 2006).

Colours such as blue, green, violet are produced by the reflection of different light wavelengths by microstructures, the distances and dimensions of these structures playing an important role in colour generation. Thus, for some species, lower distances and widths of chitin structures correspond to the green-blue (450–570 nm) colour, while higher values correspond to violet (380–450 nm) (Mika et al. 2012). Explicit correlations between attributes of structures and colour were proved by Bálint et al. (2012) using artificial prediction methods and colour models. The influence of the perforation factor on manipulation of light wavelengths was also showed for Lycaenidae species (Vértsey et al. 2004, Wilts et al. 2009).

Within the Lycaenidae group, the wing microstructures belong to the *Urania* type, but with some particular aspects. The lycaenid scales possess longitudinal ridges, connected by ribs, with perforated layers underneath them. The perforations resemble in shape and disposition with the arrangement of pepper seeds in a pot - „pepper-pot structure” (Tilley & Eliot 2002).

Researches (Wilts et al. 2009) showed that, at least in Theclinae and Polyommatae, the differences in wing reflectance and, thus, in wing colours (hues) are the result of several factors. Among these, the number of perforated layers, the width of layers and the distance between them, the degree of perforation and shape of perforations and ridge height were indicated. Kertész et al. (2006) demonstrated that hole diameter, width of walls between holes and filling factor are different among distinct genera and even among species of the same genus. The reflectance values of scales are species-specific, the reflectance constituting therefore a parameter that can be used to differentiate taxa (Piszter et al. 2011).

In researches such as Jiggins et al. (2001) or Bálint et al. (2007) it is suggested that differences in wing colours play an important role in prezygotic species isolation, even in species that are genitally similar, and are flying in the same areas (sintopically) and in the same time (synchronously). This phenomenon was also described by Kertész et al. (2006) and by Bálint et al. (2012) in the case of *Polyommatus* species.

Although several studies have been concerned

with species of the Polyommatae, the literature surveyed doesn't include species from the *Cupido* genus. In Romania, the *Cupido* genus is represented by five species, *C. argiades*, *C. decoloratus*, *C. alcetas*, *C. minimus* and *C. osiris*. *C. minimus* males have brown upper side of wings, while in the other species males have blue upper sides. *C. decoloratus* and *C. alcetas* have similar patterns on the underside of wings. However, the males of *C. argiades* (Pallas, 1771), *C. decoloratus* (Staudinger, 1886), *C. alcetas* (Hoffmannsegg, 1804) show different hues of wing colours. Furthermore, the mentioned species have overlapping flight periods and similar geographical distribution (Carter & Hargreaves 1988, Higgins & Riley 1988). Therefore, we consider that a micromorphological study on these species might reveal interspecific differences at this level.

Material and methods

The investigated material was represented by male individuals of three species of the *Cupido* genus: *C. decoloratus*, *C. alcetas* and *C. argiades*. Individuals from each species were collected during the 2012 season, from natural reservations from Romania, in the N-E of the country, near Iasi county (Fânețele Seculare de la Valea lui David, Săraturile de la Valea Ilenei, Poiana cu Schit, Pădurea Pietrosu and Pădurea Uricani).

Forewings were prepared for scanning electron microscopy on a Vega II SBH Tescan SEM analyses by placing on double-sided carbon tape and sputtering with Au layer (15 nm thickness). The SEM analysis, among other microscopic techniques, is a very valuable method for analysing insect external morphology (Çakic and Ergen 2012). The microscope was operated at magnifications up to 50,000x. From each species, wings from five individuals (the number of individuals was limited by the rare and endangered status of *C. alcetas*) were used for microscopic observations. Scales with structural colours were identified by the presence of the pepper-pot structures under the superficial ridges and ribs.

In order to assess interspecific differences at the level of scales, measurements of chitinous structures were performed. The distances between longitudinal ridges and between transversal ribs were calculated with the Atlas software included in the SEM utilities, on three scales from each individual of the three species. For each scale, five distances between ridges (Drid) and fourteen distances between ribs (Drib) were measured (Fig. 1), obtaining respectively 75 and, 210 measurements per species. Statistical analyses were performed in MS Excel by obtaining means and standard errors and by analysis of variance (ANOVA).

The number of holes contained within scale microcells was calculated using ImageJ software. The microcells are considered delimited by two consecutive ridges

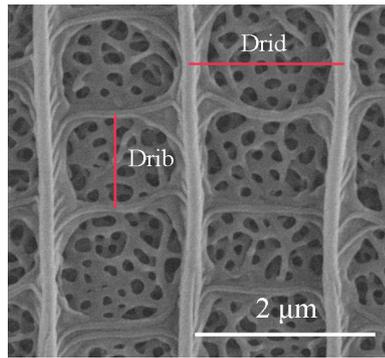


Figure 1. Distance measurement on scale microcell.

and two consecutive ribs. The same software was used to calculate the “filling factor” (Kertész et al. 2006) of the cells.

Undamaged cells were selected for measurements, with the upper layer of perforations intact. Initially, the images obtained from SEM were converted into black and white images. The number of holes was counted by selecting visible holes beginning with the topmost layer, using the ImageJ particle analyzer feature with the transformed images. The filling factor, regarded as the ratio of chitin to holes, was calculated by selecting cells without ridges and ribs, and dividing the number of white pixels (considered as chitin) by the number of black pixels (considered as holes). For number of holes and for cells filling factor, six cells per individual per species were analysed (30 cells per species). Results are presented as mean and standard error.

The interrelation among the four above investigated parameters was assessed through a Principal Component Analysis. The number of observations was $n=30$. For filling factor and number of holes, the data was used as recorded through measurements, while for the distances between ridges and between ribs, means were used. The test was run with the aid of XLStat software. The Principal Component Analysis is a method, among other statistical uses, used in determining patterns of morphological variability, that can evaluate relations between numerous parameters (Torres et al. 2013).

Results

Following the SEM data acquisition, parameters of scales were quantitatively analysed. SEM sample images, along with specimen wings are illustrated in Fig. 2 for the three species investigated. Distances between adjacent ridges and ribs forming microcells are shown in Table 1, together with the ratio of the dimensions. In the case of selected cells, the number of perforations in upper layer and the ratio of chitin/perforations (filling factor) are given in Table 3.

The species investigated by us, *Cupido argiades*, *C. decoloratus* and *C. alcetas* show specific values for the analysed parameters. The distances between ridges present higher values in *C. argiades* and *C. alcetas*, with *C. decoloratus* showing lower values than the other two species. The distances between ribs follow a slightly different pattern, with closer values in *C. argiades* and *C. decoloratus*, while *C. alcetas* records the highest values. By analysing the ratio between the two distances, the shape of the cell delimited by two consecutive ridges and two consecutive ribs can be estimated. The shape of the cell appears rectangular in *C. argiades* and *C. decoloratus* (2.33 and 2.32 ratio), while the cell has a squared shape in *C. alcetas* (1.48).

Another parameter analysed was the average number of holes in scale microcells. The investigated species present heterogeneous values of this parameter, revealing variability among species. *C. alcetas* records the highest number of holes, 33.3 per cell, followed by *C. argiades*, with 30% less holes per cell and *C. decoloratus*, with over 40% less holes. Regarding the “filling factor”, representing the ratio of chitin to holes, the three species record a degree of variability. *C. decoloratus* shows the highest values of the filling factor, 3.24, while *C. argiades* and *C. alcetas* have a filling factor below 3. Correlating the two parameters described above, we observe that *C. decoloratus* presents, as expected, the highest filling factor which corresponds to the least number of holes. However, the other two species elude this rule. *C. alcetas*, although with more holes per cell than *C. argiades*, presents a higher filling factor.

The Principal Component Analysis of the four above investigated parameters revealed correlations between variables (Table 5). The distance between ridges is correlated with the distance between ribs (0.394) and with the number of holes in a positive manner, while it is negatively correlated with the filling factor. The distance between ribs shows a stronger positive correlation with the number of holes (0.459), while a negative correlation is found between the filling factor and the number of holes.

The percentage of variability comprised in computed factors is highest for F1 and F2 respectively, with 46.91% and 23.79% of the total variability (Table 5). The following plots were therefore realized using those two factors as axes.

The factor loadings indicate that the first factor is most powerful correlated with distance between ribs, between ridges and with number of

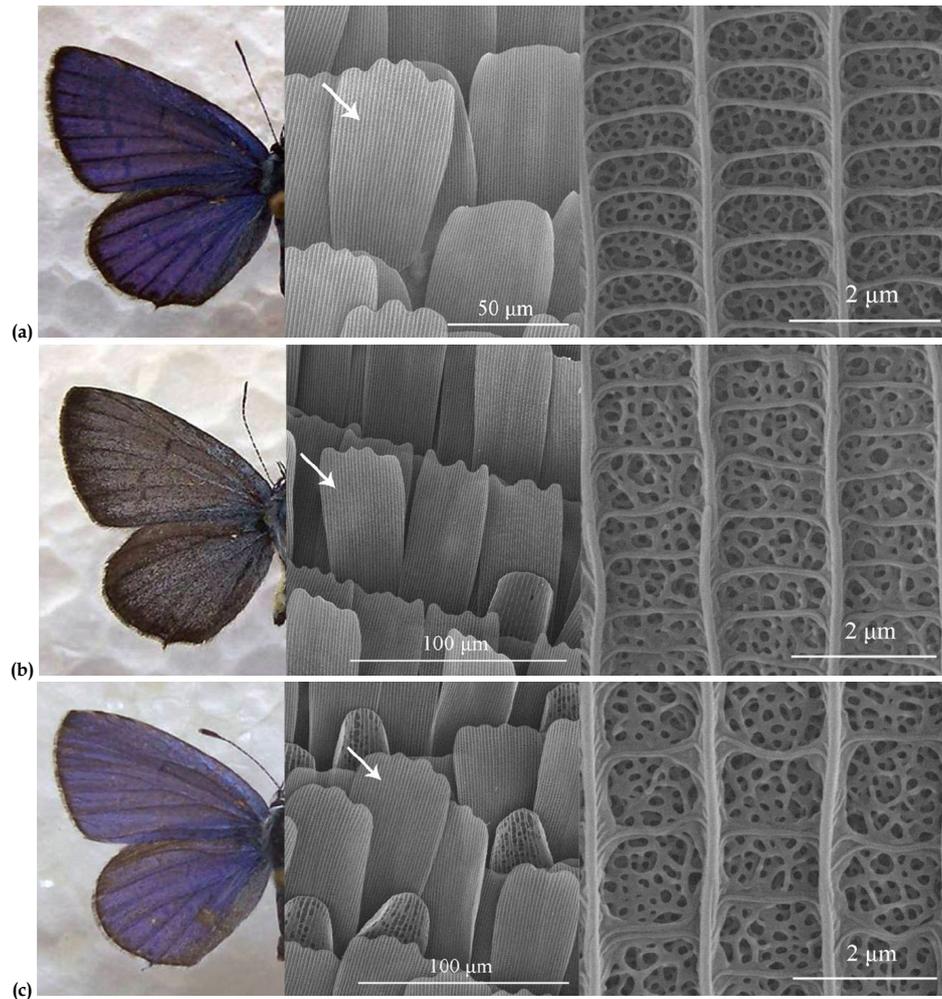


Figure 2. Wings and wing scales aspects of three *Cupido* species (a - *C. argiades*, b - *C. decoloratus*, c - *C. alcetas*).

Table 1. Dimensions of scale distances in investigated species (Drid - distance between ridges, Drib - distance between ribs, values in the same column are significantly different as shown by ANOVA for $p < 0.01$).

Species	Drid (N=75)	Drib (N=210)	Drid/Drib ratio
<i>Cupido argiades</i>	1.71 (ISE: 0.018)	0.73 (ISE: 0.007)	2.33
<i>Cupido decoloratus</i>	1.40 (ISE: 0.017)	0.60 (ISE: 0.006)	2.32
<i>Cupido alcetas</i>	1.63 (ISE: 0.015)	1.10 (ISE: 0.020)	1.48

Table 2. Analysis of variance (ANOVA) results for distances between ridges and ribs.

Parameter	F	Fcrit	P value	SS between groups / within groups	MS between groups / within groups
Drid (N=75, df=224, $p < 0.01$)	88.39717	4.702035	5.78E-29	3.760364/4.721875	1.880182/0.02127
Drib (N=210, df=629, $p < 0.01$)	374.9826	4.63916	7.8E-108	27.96052/23.37607	13.98026/0.037282

Table 3. Cells number of holes and filling factor values in investigated species (values in the same column are significantly different as shown by ANOVA for $p < 0.01$)

Species	No. of holes (N=30)	Filling factor (N=30)
<i>Cupido argiades</i>	22.66 (ISE: 0.682)	2.11 (ISE: 0.073)
<i>Cupido decoloratus</i>	18.70 (ISE: 0.867)	3.24 (ISE: 0.185)
<i>Cupido alceas</i>	33.30 (ISE: 1.495)	2.54 (ISE: 0.102)

Table 4. Analysis of variance (ANOVA) results for hole areas and filling factor

Parameter	F	Fcrit	P value	SS between groups /within groups	MS between groups /within groups
No. of holes (N=30, df=89, $p < 0.01$)	49.49763	4.85777	4.42E-15	3419.622/3005.267	1709.811/34.5433
Filling factor (N=30, df=89, $p < 0.01$)	19.320875	4.85777	1.14E-07	19.45176/43.79468	9.72588/0.503387

Table 5. Correlation matrix and Principal Component Analysis results

Correlation matrix (Pearson (n)):				
Variables	Drid	Drib	Fill. fact.	Holes nr.
Drid	1	0.394	-0.322	0.225
Drib	0.394	1	-0.128	0.459
Fill. fact.	-0.322	-0.128	1	-0.189
Holes nr.	0.225	0.459	-0.189	1

Values in bold are different from 0 with a significance level $\alpha = 0.05$

Principal Component Analysis:				
Eigenvalues:	F1	F2	F3	F4
Eigenvalue	1.876	0.952	0.714	0.458
Variability (%)	46.912	23.792	17.843	11.453
Cumulative %	46.912	70.704	88.547	100.000

Table 6. Eigenvectors and factor loadings of PCA.

Eigenvectors:	F1	F2	F3	F4
Drid	0.525	-0.297	0.648	-0.465
Drib	0.561	0.411	0.225	0.683
Fill. fact.	-0.385	0.752	0.453	-0.285
Holes nr.	0.512	0.421	-0.570	-0.486

Factor loadings:	F1	F2	F3	F4
Drid	0.719	-0.290	0.547	-0.315
Drib	0.768	0.401	0.190	0.462
Fill. fact.	-0.528	0.733	0.383	-0.193
Holes nr.	0.701	0.411	-0.482	-0.329

holes. The second factor is stronger correlated with the filling factor (Table 6).

The placement of the species in relation with the chosen factors can be observed from the individual observations scores suggesting that the three species are, to some degree, disjunct, as shown in Figure 4.

Discussion

Among variable characters in Lycaenidae morphology, one obvious and also intriguing trait is wing colour. It is known that different hues are

generated by different micromorphological structures included in wing scales. This is known for species such as *Polyommatus sp.*, *Celastrina argiolus*, *Plebejus icaroides*, *Callophrys dumetorum* (Vértesy et al. 2004, Prum et al. 2006, Bálint et al. 2007, Wilts et al. 2009). Differences in these species at scale microstructures level are described as both qualitative and quantitative. Such studies were conducted on species of other butterflies families as well, leading to the conclusion that differences in wing colours in Nymphalidae and Papilionidae (Wickham et al. 2006, Mika et al. 2012), Nymphalidae (Matějkov-Plšková et al. 2010), Uraniidae (Prum et al. 2006), Pieridae (Wilts et al. 2011) are

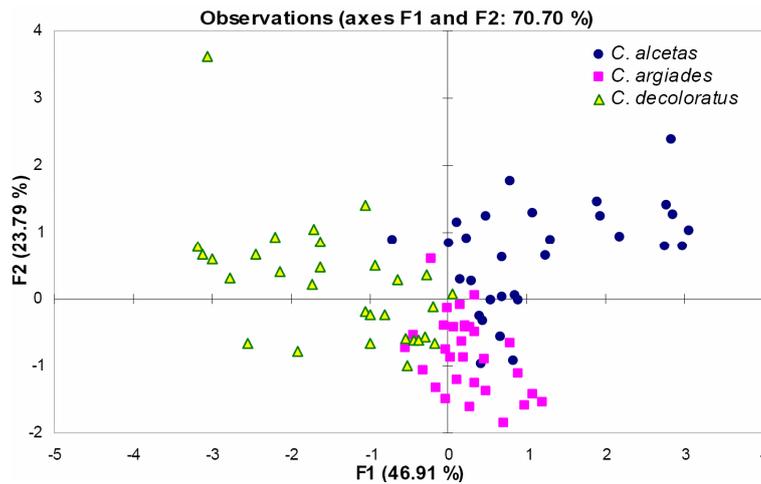


Figure 4.
PCA observations biplot.

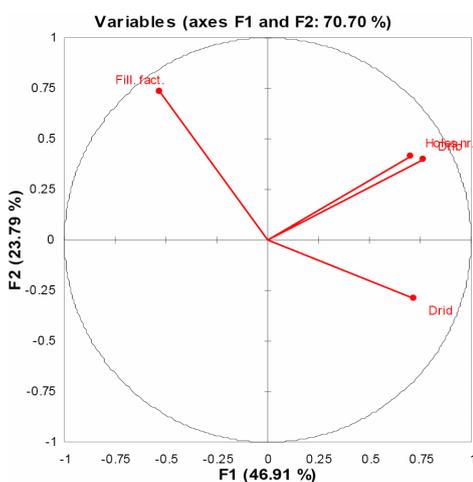


Figure 3. Principal Component Analysis correlation circle of variables.

generated by different scale morphological features.

The differences found in the analysed parameters of the three *Cupido* species investigated show a statistical significance revealed through analysis of variance testing (Table 2, 4), thus they indicate a preliminary basis for species discrimination which may complement other morphological criteria. This possibility was already hypothesized for other lycaenid species (Bálint et al. 2007), where similar measurements of scale structures, combined with spectroscopic data, allowed for clear differentiation of five *Polyommatus* species.

Our results are in agreement with those obtained by Tilley and Eliot (2002), who record a

mean distance between ridges of 1.5 μm in the scales of a *Cupido* species, *C. minimus*. The *Cupido* species analysed by us possess a distance between ridges of 1.4-1.7 μm . In comparison with other Polyommatae species, the species investigated by us have intermediate values for the analysed parameters, as Wilts et al. (2009) find values of 1.3-2.5 μm for distances between ridges and of 0.6-1.9 μm for distances between ribs in four Polyommatae taxa (*Celastrina argiolus*, *Danis danis*, *Plebejus icarioides*, *Polyommatus icarus*). Similarly, Bálint et al. (2007) find distances of 1.4-1.76 μm between ridges and of 0.56-1.0 μm between ribs in five *Polyommatus* taxa (*Polyommatus eroides*, *P. eros*, *P. erotides*, *P. ariana*, *P. icarus*). The same species possess a filling factor of 1.96-5.77, with specific values for investigated species.

The perforation factor, known also as filling factor, is demonstrated to relate closely to another parameter used to differentiate among species, the reflectance. The peak of reflectance of light from a structural coloured scale decreases with the increase in perforation degree (Wilts et al., 2009). This idea was proved for Uraniidae and Papilionidae taxa by Prum et al. (2006) who demonstrates also the role of periodicity of microstructures in coherent scattering of light by butterfly scales. For lycaenid species, works such as those of Kertész et al. (2006) or Vértésy et al. (2006) record values for reflectance and filling factors sustaining the relation of inverse correlation between these two parameters. The filling factor and dimensions of scale microcells were shown to be one of the most important factors in establishing reflectance values in artificial models, possibly in real models too

(Bálint et al. 2012). The analysed species present interspecific variability at the level of the investigated parameters (distances between ribs and between ridges, filling factor), variability proved statistically significant. Values obtained fit those recorded by other authors for Polyommatinae species, while bringing new data for analysed species. Differences observed may constitute a basis for further investigations on physical and optical properties of considered species scales, with possible taxonomical value.

The link between the analysed variables can be seen in Figure 3, with distance between ridges and the filling factor in an inverse correspondence, while a positive relation is present between number of holes and distance between ridges and ribs. The variables which most powerful influence the number of holes are the distance between ribs and between ridges. The results indicate that the greater the size of the microcell, the greater the holes number. In the same time, the filling factor should decrease with the distance between ridges, therefore with the size of the microcell. This trend is expectable, as the filling factor is represented by the ratio of area occupied by chitin to the occupied by the holes.

Considering the position of the species as related to the two principal factors (F1 and F2), a degree of separation of the species can be seen (Fig. 4), with *C. decoloratus* being more isolated than the other two species in respect to distance between ridges, ribs and number of holes. In the meantime, *C. argiades* is farther than *C. decoloratus* especially with regard to the filling factor.

Such results further emphasize the variability that occurs at micromorphological level in lycaenid species, and the relations that exist between different parameters of scales. Combining this kind of data with measurements of other optical and physical parameters may prove useful in characterization of different species. A more detailed modelling of scales will, as well, enable a better understanding of structures of scales function and evolution.

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*** ImageJ (Image Processing and Analyzing in Java): available at: <http://imagej.nih.gov/ij/index.html>