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Quantitative description and discrimination of butterfly wing patterns using moment invariant analysis

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Abstract

Studies examining and using pattern variation in insects for identification and characterization of individuals and populations have been limited by the methods available for quantifying wing patterns objectively. In this paper, differences in wing pattern are demonstrated statistically using moment invariant data sets generated automatically from digitized images of the speckled wood butterfly, *Pararge aegeria* (Linnaeus). Studies with other biological subjects have already shown moment invariants to work well with outline shapes and silhouettes. A pilot study with replicated monochrome photographs of a single butterfly showed the method could detect pattern differences between wing surfaces, even in the presence of simulated wing fading and damage. In a further study of the wings of 228 specimens, multivariate analyses of variance using the moment data reliably detected differences between groups of butterflies according to sex, geographical origin and culture history. Potential applications and future improvements of the moment methodology are considered.

Introduction

Biological studies frequently require the measurement of variation among individuals. Although molecular techniques are available, it can sometimes be more informative to quantify and interpret phenotypic variation such as surface patterns. The measurement of pattern has often been limited by the use of manual methods or by image analysis procedures tailored to specific image features. To describe the shapes and patterns of organisms, biologists traditionally examine many specimens to define characters representing some features of the variation and to record their values for analysis (Rohlf, 1990). Examples of such studies in Lepidoptera include White (1974), Jarvinen & Vepsäläinen (1979), Robertson (1980), Brakefield & Liebert (1985) and Winokur (1989).

In traditional manual methods, both the recognition of pattern features and measurement of the characters defined to represent them require human intervention. However, manual data acquisition can be time-consuming, tedious and error-prone. The choice of characters is difficult, arbitrary and liable to influence the results, and the characters may be recorded inconsistently. Semi-automatic methods exist which carry out the measurements automatically, but still require the manual recognition of the features to be measured, for example by using a digitizer pad to trace feature outlines or a mouse to select landmarks which represent characteristic homologous points (Rohlf, 1990; Yu *et al.*, 1992; Meacham, 1993). The automatic location of features, though possible (White & Prentice, 1988; White *et al.*, 1988), is difficult to program, may be unreliable or incomplete, and must be redesigned and reprogrammed for each new class of object.

These difficulties can be overcome by extracting descriptors from the image that do not rely on the explicit identification of landmarks and therefore enable fully automatic feature recognition and measurement by an image

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analyser. They can describe global properties of the study object and be used routinely in studies of different types of object without reprogramming.

One such approach utilizes moment invariants to describe the shape of objects (appendix 1). These moments have been used successfully to distinguish similarly-shaped tree leaves of two closely related species, varying also with respect to environment and within-tree position (White & Prentice, 1988; White *et al.*, 1988). Other authors used moments to discriminate fish species (Strachan *et al.*, 1990; Zion *et al.*, 1999).

Moments, however, can also be generalized to describe surface patterns and texture, by which we refer to the spatial distribution of colour and brightness in images of two-dimensional objects, rather than simply to the outline or silhouette of an object to be measured. To our knowledge, this approach has not been applied in biology, but has the potential to provide a reliable method for quantifying wing patterns with a number of advantages. It can be used for identification, age assessment and other applications.

This paper demonstrates an automatic method for measuring pattern from wing images. First, photographs of the wings of a single specimen of the speckled wood butterfly, *Pararge aegeria* (Linnaeus) (Lepidoptera: Satyridae), are used to test the method and the effects of simulated wing damage and fading, and then the method is used to demonstrate differences between individuals from an earlier study (Winokur, 1989).

Materials and methods

Test photographs

The replicated monochrome photographs depict an undamaged male (fig. 1). The four detached wings were photographed together in both their dorsal and ventral views. To avoid confusion with references to the eight individual wing surfaces, 'surface' in the context of dorsal and ventral differences will be termed 'aspect'.

Twenty-eight replicate pairs of photographs (one dorsal and one ventral photograph per 'replicate') were printed. Of these, eight 'density' replicates were under-exposed during printing to varying degrees to reduce their density and so simulate the overall scale loss ('rubbing') and fading which affect adult butterflies as they age. The density replicates were numbered on a scale from 1 (printed normally) to 8 (least dense). The other 20 replicates were printed with equal density. Of these, ten were doctored to simulate various types of natural damage ('damage' replicates, fig. 2). Typical outlines of missing or rubbed areas of forewings and hindwings were traced from photographs of various other individuals (Winokur, 1989), and superimposed on to the appropriate wing of the dorsal and ventral photograph of each respective replicate. As far as possible, similar kinds of damage were applied to both forewings and hindwings of each replicate pair. The area corresponding to the damage was then deleted with typing correction fluid to appear as background. The remaining ten replicates were unmodified and served as controls ('control' replicates).

Image acquisition

Image acquisition was achieved using a computer system with a monochrome video camera. Dorsal and ventral

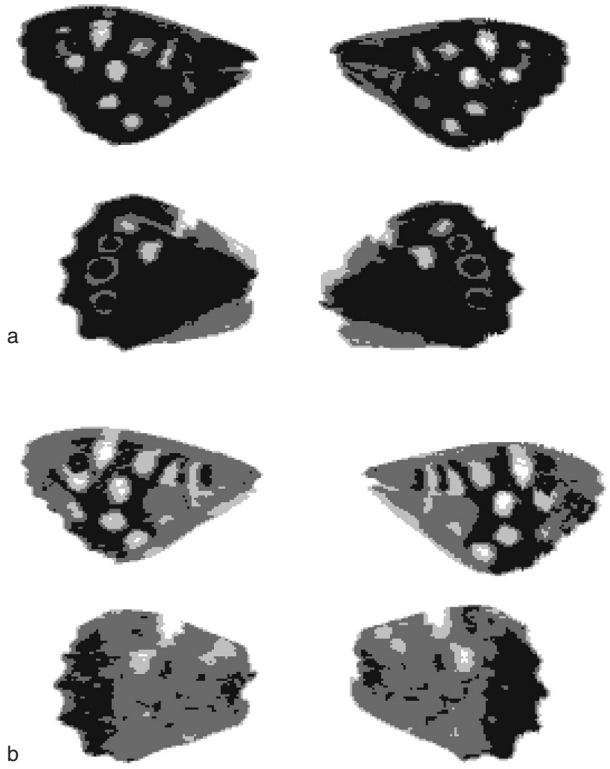


Fig. 1. Digitized images of one of the control replicates of *Pararge aegeria* specimen 01 002 003 collected in Southampton in April 1985 (Winokur, 1989): (a) dorsal aspect; (b) ventral aspect.

images were captured and transferred to image files through a video camera interface. The images were separated into the eight image sections comprising the dorsal and ventral aspects of the left and right forewings and hindwings as shown in fig. 1. The object is represented within a rectangular array of small integers, each representing the brightness of a square 'pixel' of the image. In the present study a grid resolution of 320 pixels (horizontal) by 192 (vertical) was used. Four brightness levels were calibrated to distinguish the grey levels corresponding to the white of the eyespot pupils, cream and orange markings, and dark brown background. Scanners and digitizers currently available permit higher resolutions, but a digital camera or a video system such as that used here has the advantage of being able to capture images directly from specimens of any size.

Pattern measurement

The use of spatial brightness distributions was anticipated in some treatments (Hu, 1962), but most applications of the moment method have assumed just two brightness levels corresponding to the object and its background (Dudani *et al.*, 1977) and use either those image pixels that coincide with the object silhouette (Rohlf, 1990) or outline (White & Prentice, 1988; White *et al.*, 1988). The present study used all pixels within the wing image area, but to accommodate the brightness information, each pixel's contribution in the moment formulae was weighted in proportion to its darkness value (appendix 1). White pixels

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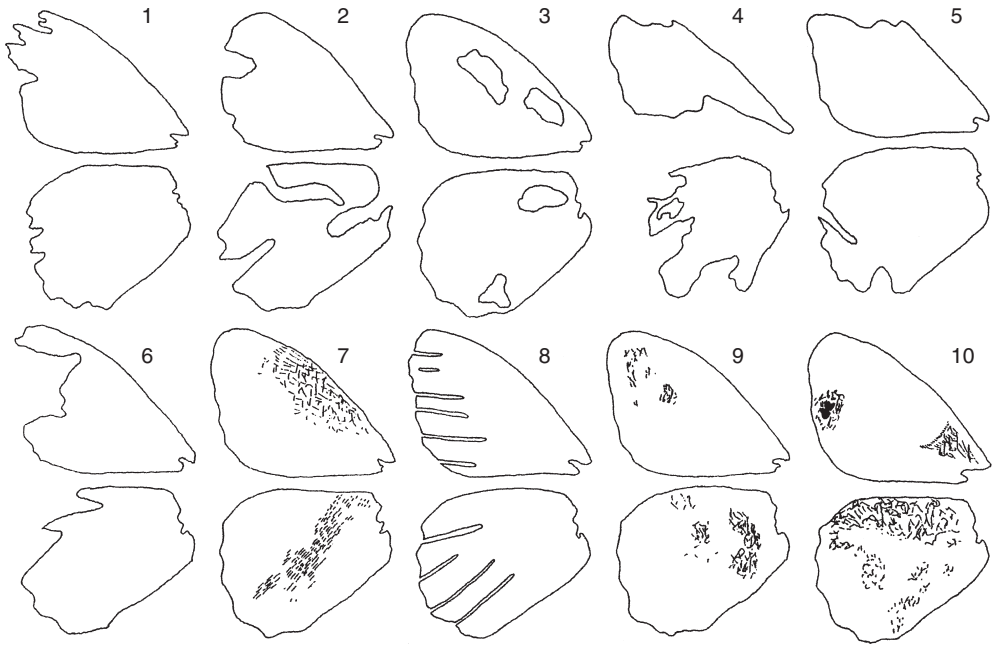


Fig. 2. Artificial wing damage templates applied to the photographic replicates. Damage types 7, 9 and 10 represent localized rubbing; damage type 8 depicts rubbing along the wing venation. In damage type 10, an undamaged zone within the forewing marginal rubbed area is shown blackened to emphasize its shape.

were given the value zero. In this way the moment values described the distribution of darker areas within the wings and ignored the white background. Thus an explicit object detection algorithm was not required. The seven moment invariants were calculated for each of the eight wing images (dorsal and ventral aspects of the left and right forewings and hindwings) using the 'Arbo' program written by White. After digitizing and measuring the images, Arbo constructed a multivariate data matrix with one row of seven moment values per wing surface for subsequent statistical analysis.

Analyses

The test photographs were analysed to quantify inherent differences between the surfaces, and to assess the effects on the measurements of simulated wing damage and fading, without these being confounded with natural variation between specimens. Because the relationship of these moments to visible pattern attributes is not fully understood, no attempt was made to relate individual moment values to specific pattern attributes, but instead the seven moment values were treated as a multivariate vector, in order to test for statistically significant differences between wings and butterflies where these were expected to exist.

The first set of analyses treated each wing surface as a case for analysis with seven moments per case and eight cases per butterfly. The effects of side (left/right), wing (forewing/hindwing), aspect (dorsal/ventral) and replication on each of the seven moments were tested using univariate four-way analyses of variance. Multivariate analyses of variance (MANOVA) on the seven moments as a group used all the available pattern information to give a unified assessment of these factor effects and interactions. Principal components analysis (Sokal & Rohlf, 1981) was then performed separately for each of the dorsal and ventral forewing and hindwing surfaces, treating their left and right sides as replicates, to determine the extent to which the multivariate distribution of the 28 replicates would separate the control, density and damage replicates into discrete groups.

The analysis of differences between individuals used generally similar methods but differed in the following details. Unaltered images were captured directly from the wing surfaces of 22 samples of butterflies (Winokur, 1989) rather than from replicated images of a single individual. Analyses of variance tested the detection of differences between the wings, aspects and sides. The influence of damage was assessed not by comparing damage and density replicates with controls but by comparing three levels of damage severity, i.e. 'undamaged' (surface perfect on scrutiny = condition level 1), 'minor damage' (with minor rubs or tears but of overall acceptable appearance = level 2) and 'major damage' (with obvious scale loss or missing areas = level 3), and irrespective of the individual to which each relevant surface pertained. The detection of differences due to sample (reflecting their origin, seasonal flight period and rearing temperature), sex, and pupal chilling and darkness treatments (Winokur, 1989, 1992) and their specificities to particular surfaces were also assessed.

The effects of these latter factors were then re-examined in uni- and multivariate analyses of variance using a second approach that treated all eight surfaces of each entire butterfly as one case with 56 moment values. Since this necessitated some inclusion of damaged surfaces, the optimum level of damage acceptance for comparing its efficacy was established by testing various criteria of mean

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and most severe damage allowable on any individual. Owing to the large number of variables, univariate effects were here considered significant only if $P \leqslant 0.01$. Finally, entire butterfly analyses were performed using canonical discriminant analysis, which examines the extent to which groups of individuals can be distinguished on the basis of their locations in a discriminant space, where the position of individuals in each group is described with respect to canonical discriminant axes that best display the separation between the groups (Sokal & Rohlf, 1981). It was used for visual assessments on scatter plots of the separation between samples, the significance of which was tested by one-way MANOVA using Wilks' lambda statistic to assess the magnitude of the between-sample differences relative to within-sample (between-individual) variation in a manner analogous to the univariate F test. Finally, the two approaches of analysing the factors using the surfaces individually or collectively were compared.

All statistical analyses were performed using the SPSS^x analysis package (SPSS, Inc., 1997) and further interpretations were made by visual examination of the digitized images.

Results

Surface comparisons using test photographs

Means and standard deviations of the moment invariants are shown in appendix 2. Comparisons between the wing surfaces of *P. aegeria* using the photographic control replicates are shown in table 1a. Individual moments and the MANOVA detected forewing–hindwing and dorsal–ventral differences and interactions between wing and aspect, in accordance with visible differences (fig. 1). Since the dorsal forewing, ventral forewing, dorsal hindwing and ventral hindwing have distinct patterns, the interpretation of differences between the wings or between the aspects will need to take the other factor into account.

Differences between the sides and interactions involving side were detected. Since consistent directional asymmetry or antisymmetry (Soulé, 1967) do not occur in Lepidoptera (notwithstanding the findings of Windig & Nylin, 1999), the differences between the sides in one individual most likely result from the random effects of fluctuating asymmetry (Palmer & Strobeck, 1986) being replicated in multiple copies of the same photograph. The interactions reflect the independent expression of fluctuating asymmetry on each of the four pairs of wing surfaces (Winokur, 1996).

Photographic replication had only a small effect on moment values, except in its interaction with aspect (table 1a), which possibly reflects small differences in density between the photographs or in the alignment of the pixel grid. The relative contributions of the factors in table 1 to the variation in moment values are estimated in appendix 3.

Effects of simulated rubbing and damage using test photographs

Density level (table 1b) and simulated damage (table 1c) significantly affected all moment invariants. They also showed interactions with wing and aspect, presumably because the different patterns on each of these surfaces are degraded differently. In the case of the different damage patterns (fig. 2), the interaction with aspect arose because each comparison of a dorsal surface with its corresponding ventral surface used a different area of remaining undamaged pattern. The interaction with wing simply reflected the different damage patterns applied to the forewings and the hindwings.

Comparison of table 1b and 1c with 1a shows that both reduced print density and simulated damage reduced the detection of asymmetry and some of the interaction between wing and aspect. Indeed, they would be expected to have the greatest relative impact on the detection of low magnitude differences (as occur between the sides) and measurement of low contrast features (as on the ventral hindwing).

The different types of simulated damage applied to the forewings and hindwings added to the pre-existing differences between forewing and hindwing, and is reflected in the highly significant interactions between damage and wing (table 1c). The damages applied in each replicate to left and right wings, on the other hand, were mirror images. Hence no interaction with side was observed, and the reduced significance of differences between the sides can be understood as the equivalent obliteration on left and right of wing areas bearing the original asymmetry.

Figure 3 shows separate principal component analyses of the dorsal forewing, ventral forewing, dorsal hindwing and ventral hindwing, in which the first two principal axes explained 87.0, 86.6, 87.8 and 89.6% of the total variation in their respective moment data. The control replicates are clustered together thereby demonstrating their similarity. The replicates with progressively reduced densities and showing decreasingly discernible pattern, however, are displaced progressively to one side, while the damage replicates are more generally dispersed, indicating that the different damage types disrupted the wing pattern in less consistent ways.

Surface comparisons using individual butterflies

The results of comparisons between the wings, aspects and sides using undamaged individuals (condition level 1) of *P. aegeria* are given in table 2. Individual moments and the MANOVA detected forewing–hindwing and dorsal–ventral differences and interactions between wing and aspect, in accordance with visible differences. No side differences were detected, consistent with bilateral symmetry. Some interaction of side with wing and aspect was detected, but is understood to reflect the net fluctuating asymmetries present on the separate surfaces (Palmer & Strobeck, 1986; Winokur, 1996).

Comparable analyses using damaged surfaces show that minor damage (condition 2) reduced the significance of some effects, while major damage (condition 3) obscured all except forewing–hindwing differences and some dorsal–ventral distinctions (table 2). Both levels of damage introduced some new effects at the $P \leqslant 0.05$ level. Hence the presence of damage obscures real effects and may introduce false ones. The following analysis therefore used undamaged surfaces only.

Sample, sex and treatment differences in analyses of each surface

Four separate four-factor analyses of sample, sex, treatment and side were performed for each of the dorsal

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Table 1. Influence of surface manipulation factors on the moment invariant measurements in the photographs analyses.

Interactions and factors	d.f.	m_1	m_2	m_3	m_4	m_5	m_6	m_7	MANOVA
(a) 10 control replicates									
Wing × aspect × replicate	9, 36							*	
Wing × replicate	9, 36								
Aspect × replicate	9, 36	***		*	***				***
Side × replicate	9, 36								
Wing × aspect × side	1, 36	***	***		***	***	*		***
Wing × aspect	1, 36	...	...	***	...	...	***		...
Wing × side	1, 36		...	***	.		**	***	...
Aspect × side	1, 36		...	***	...	...	***	*	...
Wing	1, 36	...	...	...	...			...	...
Aspect	1, 36	...	...	...	...	...	...	***	...
Side	1, 36	...	...	...	...				...
Replicate	9, 36	..		.					.
(b) 8 density levels									
Wing × aspect × density	7, 28			**		*			***
Wing × density	7, 28		***		*				...
Aspect × density	7, 28		***	***	***	***			...
Side × density	7, 28								
Wing × aspect × side	1, 28		*					*	***
Wing × aspect	1, 28		**				*		...
Aspect × side	1, 28		..	.					
Wing × side	1, 28				*				...
Wing	1, 28	**	...		***	***	**		...
Aspect	1, 28	***	..	.	...	...	***	***	...
Side	1, 28								
Density	7, 28	***	...	...	...	...	***	***	...
(c) 10 damage types									
Wing × aspect × damage	9, 36	**	**	**	***	*		**	***
Wing × damage	9, 36	***	***	***	...	***	***	***	...
Aspect × damage	9, 36		***		...	.		..	..
Side × damage	9, 36								
Wing × aspect × side	1, 36		***					**	***
Wing × aspect	1, 36	***	...		***	***	***	..	...
Aspect × side	1, 36				*	*		..	...
Wing × side	1, 36								
Wing	1, 36	...	...	...	...	...	...	...	...
Aspect	1, 36	...	...	***	...	...	...	***	...
Side	1, 36	*	.	*					..
Damage	9, 36	...	...	...	...	...	...	...	...

Four-way analyses of variance for each of the three series of replicates (a, b and c) described in appendix 2 were performed using the regression method of sums of squares decomposition, and the four-way interaction and three-way interactions involving both side and replicate were pooled and used as the error term. Columns labelled m_1 to m_7 refer to analyses of variance of individual moments. The MANOVA analyses tested the significance of the main effects and interactions on overall wing morphology using all seven moments together. Significance level conventions used here and in tables 2, 4 and 5 and appendix 4: * $0.01 < P \leqslant 0.05$, ** $0.001 < P \leqslant 0.01$, *** $P \leqslant 0.001$; empty cells denote non-significant results; dots describe the significance of effects included in significant higher-order interactions.

and ventral forewings and hindwings (table 3). Individual moments and MANOVA detected sample differences on all surfaces except the ventral hindwing, consistent with subspecies differences (Tolman & Lewington, 1997), seasonal trends (Robertson, 1980) and interaction between temperature and ancestral treatment (Winokur, 1992). m_2 detected sexual dimorphism on the dorsal hindwing reflecting the more prominent pale markings and eyespots of females; this further interacted with sample but the relationships were not visibly obvious.

Effects of pupal chilling and darkness were detected, inviting visual examination of the digitized images. Darkness resulted in larger pale markings, more apparent in females, an effect similar to that of shortening day-length during the larva (Shreeve, 1985). Chilling caused major aberration in a number of specimens, mainly males, and one cool-reared offspring showed more pronounced pattern aberration implicating epigenetic inheritance (Winokur, 1992; Vines, 1998). Female phenotype, seasonal forms and comparable aberration are depicted in Winokur (1995).

Sample, sex and treatment differences using multi-way analyses of entire butterflies

When all 228 individuals were used, with all available surfaces whatever their condition, no significant effects were found. The analysis was therefore re-run using the 118 individuals with all wing surfaces present and no worse than minor damage, which disclosed 17 univariate effects and sample differences in the MANOVA (appendix 4a).

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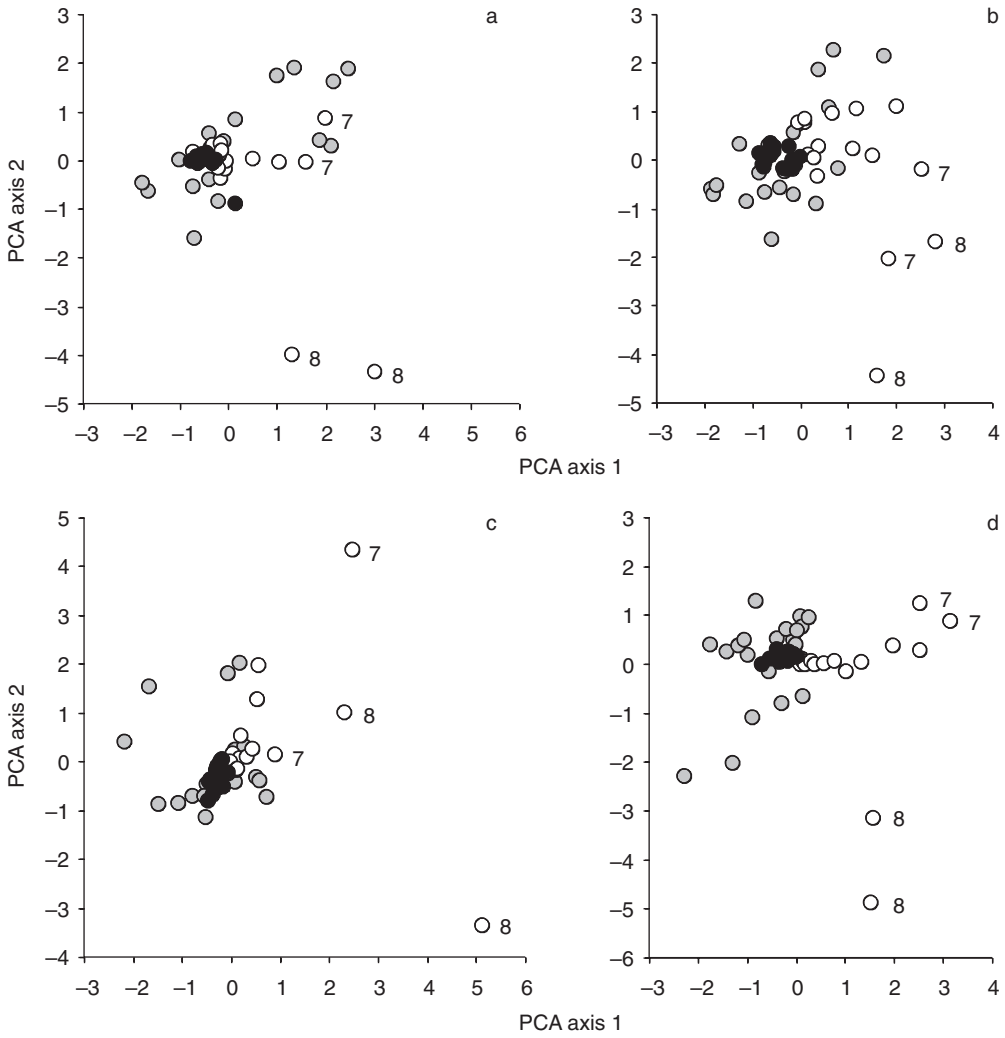


Fig. 3. Principal component analyses of the moment values for control, damage and density photographic replicates. Four analyses were performed, one for each of the dorsal forewing (a), ventral forewing (b), dorsal hindwing (c) and ventral hindwing (d). The location with respect to principal axis 1 (horizontal) and 2 (vertical) of the control replicates (●), damage replicates (◍) and density replicates (○) is shown. The two lightest density levels are indicated by '7' and '8'.

Increasingly stringent criteria of damage exclusion were thus examined using individuals with no worse than minor damage and m_1 values representative of fully expanded wings. This selection was first restricted to the 90 individuals possessing at least four perfect surfaces, which increased the number of univariate effects and the significance of sample differences in the MANOVA (appendix 4b). Further restriction to the 74 individuals with at least five perfect surfaces was then attempted but rendered the data set too small to run the MANOVA.

A fifth test was then performed with the above moment value selection criteria but with surfaces in any condition provided mean condition was less than 2.0. Few univariate effects were detected using these 149 individuals and the sample differences in the MANOVA were barely significant (appendix 4c). Further interpretation and the canonical discriminant analysis below will thus use the 90 individuals described above.

Appendix 4b shows the clear detection of sample differences in the MANOVA to be reflected in both the number and range of individual moments showing significant differences. Although one or two moments showed significant sexual dimorphism, the MANOVA failed to do so, likely reflecting the limited number of moments involved and their surface specificities.

Canonical discriminant analysis using entire butterflies

The similarities of groups classified by one or more of sample, sex and treatment were assessed by visual inspection of scatter plots that used the first two canonical axes. The significance of the discriminations was tested by one-way MANOVA, which can be regarded as a test of the ability of the moment method to differentiate between the groups.

All separations were highly significant. Visual inspection

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Table 2. Differences and interactions among the wing surfaces for each of the condition levels described in the text.

Interactions and factors	Condition	m_1	m_2	m_3	m_4	m_5	m_6	m_7	MANOVA
Wing × aspect × side	1								
	2							*	
	3								
Wing × aspect	1		***		***	**	**	***	***
	2		**		*		*		***
	3								
Wing × side	1						**		
	2								
	3								
Aspect × side	1				**	***	***		***
	2				***	***	***		***
	3								
Wing	1	***	…	***	…	***	***	…	…
	2	***	…	***	…	***	…	***	…
	3	***	***		***	**	***	***	***
Aspect	1		…		…	…	…	…	…
	2		..		…	…	…	***	…
	3	*				*			*
Side	1								
	2								
	3								

Degrees of freedom for condition level 1 (undamaged): 1 and 756 for individual moments, 7 and 750 for MANOVA; level 2 (minor damage): 1 and 631 for individual moments, 7 and 625 for MANOVA; level 3 (major damage): 1 and 413 for individual moments, 7 and 407 for MANOVA. Significance level conventions as in table 1.

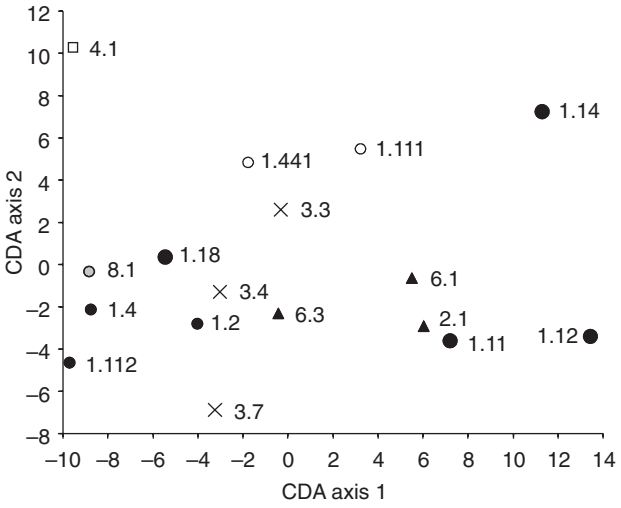


Fig. 4. Canonical discriminant plot showing the centroid locations of the 17 samples (with pupal treatments and sexes pooled) examined by one-way MANOVA (table 4). □, French subspecies *Pararge aegeria aegeria* first generation, all other samples British subspecies *P. a. tircis;* ○, south England early spring emergence; ◍, south England late spring emergence; ● (small), south England early summer emergence. Remaining samples reared under long photoperiod: ▲, north England; ×, north × south England hybrids; ● (large), F_2 and F_3 families derived from south England F_1 cold-treated parents (1.12 and 1.14) and control parents (1.11 and 1.18).

of the plots showed the 17 sample means to be well separated (group overlap = 3.3%, table 4) with greater association between samples of similar origin and history (fig. 4). On axis 2, with the exception of samples 1.14 and 1.18, progressively smaller values were taken by French *P. a. aegeria* Linnaeus, English early spring, late spring, and early summer samples (or samples reared under equivalent photoperiod) of *P. a. tircis* Butler. This accords with a north–south cline and northern European seasonal trend towards progressively smaller pale markings in the outer two thirds of the dorsal pattern, related to background matching (Robertson, 1980; Shreeve, 1985; Brakefield & Shreeve, 1992). On axis 1, early spring *P. a. tircis* showed greater values than southern England early summer samples, consistent with darker wing bases to improve solar heat absorption in the coolest season (Shreeve, 1985). Similarly, among early summer butterflies (and those reared under long photoperiod), northern samples took larger values than those from southern England, consistent with the same adaptation to improve heat absorption in cooler latitudes (Brakefield & Shreeve, 1992). Sample 1.11 reared at the coolest temperature and samples 1.12 and 1.14 of cold-treated ancestry took the largest axis 1 values, suggestive of a comparable phenotypic response.

The sexes were well separated (group overlap = 1.1%, table 4) demonstrating efficient detection of the visible sexual dimorphism. The three pupal treatments were clearly separated when used as the sole classification factor (group overlap = 4.4%), but less so when groups were classified by both treatment and sex (group overlap = 12.2%). To confirm the significance levels despite the low error degrees of freedom and the chance occurrence of atypical individuals in small samples, a randomization test based on the analysis of the 17 samples was performed, which showed that values of F (calculated from lambda) greater than or equal to 1.31 occurred with a frequency (probability) of 0.05. On the basis of this critical F value, all canonical separations were significant except the one with three treatment groups. The latter separation nonetheless represents better discrimination than was possible by eye, when only a few individuals with either overall or distinctive local pattern modification were distinguishable.

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Table 3. Summary of significant effects and interactions among the six factors tested by four-way analyses of each surface. Only undamaged wing surfaces were used, requiring the exclusion of samples 1.1, 1.7, 1.10, 1.221 and 3.2 (17 Hampshire and 12 Hampshire × Lincolnshire hybrid specimens) from the dorsal forewing analysis and sample 1.221 (two specimens) from the ventral forewing and dorsal hindwing analyses.

Interactions and factors	m_1	m_2	m_3	m_4	m_5	m_6	m_7	MANOVA
Sample	F, HD	hd		F, HD	fv, D	fd, FV, HD	F	F, HD
Sex		hd						
Treatment	fv						fv	
Sample × sex		HD		fd				HD
Sample × treatment				fd		fd		
Sex × treatment × side				fd				

Lower case lettering indicates effects significant at the $0.01 < P \leqslant 0.05$ level, upper case lettering effects significant at the $P \leqslant 0.01$ level, the wings and aspects showing the effects being denoted thus: F, both forewing aspects; fd, dorsal forewings; FV, fv, ventral forewings; HD, hd, dorsal hindwings; D, both dorsal wing surfaces.

Table 4. One-way multivariate analyses of variance and canonical discriminant analyses using the entire wing data for each of the 90 butterfly specimens used in appendix 4b, which excluded samples 1.1, 1.7, 1.10, 1.221 and 3.2.

Factor combination used to define groups	Number of groups	Number of significant		Wilks' lambda			Axes 1 and 2[c]	Group overlap[d]
		moments[a]	axes[b]	value	prob.			
Sample & sex & treatment	32	24	4	0.0000	0.000	***	60	1.1
Sample & sex	24	30	4	0.0000	0.000	***	53	1.1
Sample & treatment	24	24	4	0.0000	0.000	***	59	2.2
Sex & treatment	6	22	2	0.0004	0.000	***	77	12.2
Sample[e]	17	33	3	0.0000	0.000	***	61	3.3
Sex	2	13	1[f]	0.1121	0.000	***	100[f]	1.1
Treatment	3	26	1	0.0747	0.003	**	100[g]	4.4

Significance level conventions as in table 1. [a]number of individual moment variables significant at $P \leqslant 0.01$, based on univariate F tests; [b]number of canonical axes significant at $P \leqslant 0.05$, based on the multivariate Wilks' lambda criterion with probability values computed using the F approximation; [c]percentage of the between-group variance exhibited by the first two canonical axes; [d]percentage of butterflies nearer to the centroid of a group other than their own; [e]this is the analysis shown in fig. 4; [f]only one canonical axis exists; [g]only two canonical axes exist.

Subspecies differences using separate wing surfaces in multi-way MANOVA and canonical discriminant analyses

Pararge aegeria aegeria and *P. a. tircis* collected in April were grouped by subspecies, sex and surface. Multi-way MANOVA detected significant sex ($F_{7,235} = 5.876$, $P \leqslant 0.001$) and subspecies differences ($F_{7,235} = 3.174$, $P \leqslant 0.01$), and their interaction ($F_{7,235} = 4.272$, $P \leqslant 0.001$). The plots for each surface (fig. 5) show sexual dimorphism across both subspecies. Both sexes showed subspecies differences in forewing morphology, but only males showed hindwing differences. The digitized images showed females of both subspecies to have prominent hindwing eyespots. Since these markings divert predator strikes from the body (Shreeve, 1985), this observation accords with the greater time spent basking in exposed situations by southern European individuals, by spring broods in northern Europe and by females (Brakefield & Shreeve, 1992). Subspecies differences in the ventral hindwing were less clear-cut, but this may be due to their different ground-colours (orange-brown in *P. a. aegeria*, olive-brown in *P. a. tircis*) being represented by the same shade of grey. The MANOVA detected significant interaction between subspecies and surface ($F_{21,675} = 1.723$, $P \leqslant 0.05$), consistent with the different adaptive functions of the wing surfaces in the micro-habitats of the subspecies.

Discussion

Efficacy of moment invariants

The results demonstrate that the seven moment invariants used in the present study provide suitable quantitative pattern descriptors for multivariate analysis. The ability of the moments to detect differences between the aspects, which occur together on each wing and hence do not differ in size or shape, indicates that the moments utilized surface pattern comprising four greyness levels and not just the image outline or silhouette. The present results, despite the low image resolution, indicate that moment invariants can be used even in studies employing low resolution, such as those using flatbed scanners to record several specimens per scan or low specification digital cameras.

The particular method of statistical analysis, however, will depend upon the objectives and factors of interest. MANOVA and canonical analysis have an advantage over univariate analysis of variance in being able to distinguish between groups that share similar values with respect to individual moments but which differ in their joint distribution. Methods that produce graphical results are most suited to revealing the magnitude and direction of differences between groups. The ability of canonical discriminant analysis to differentiate groups, even when

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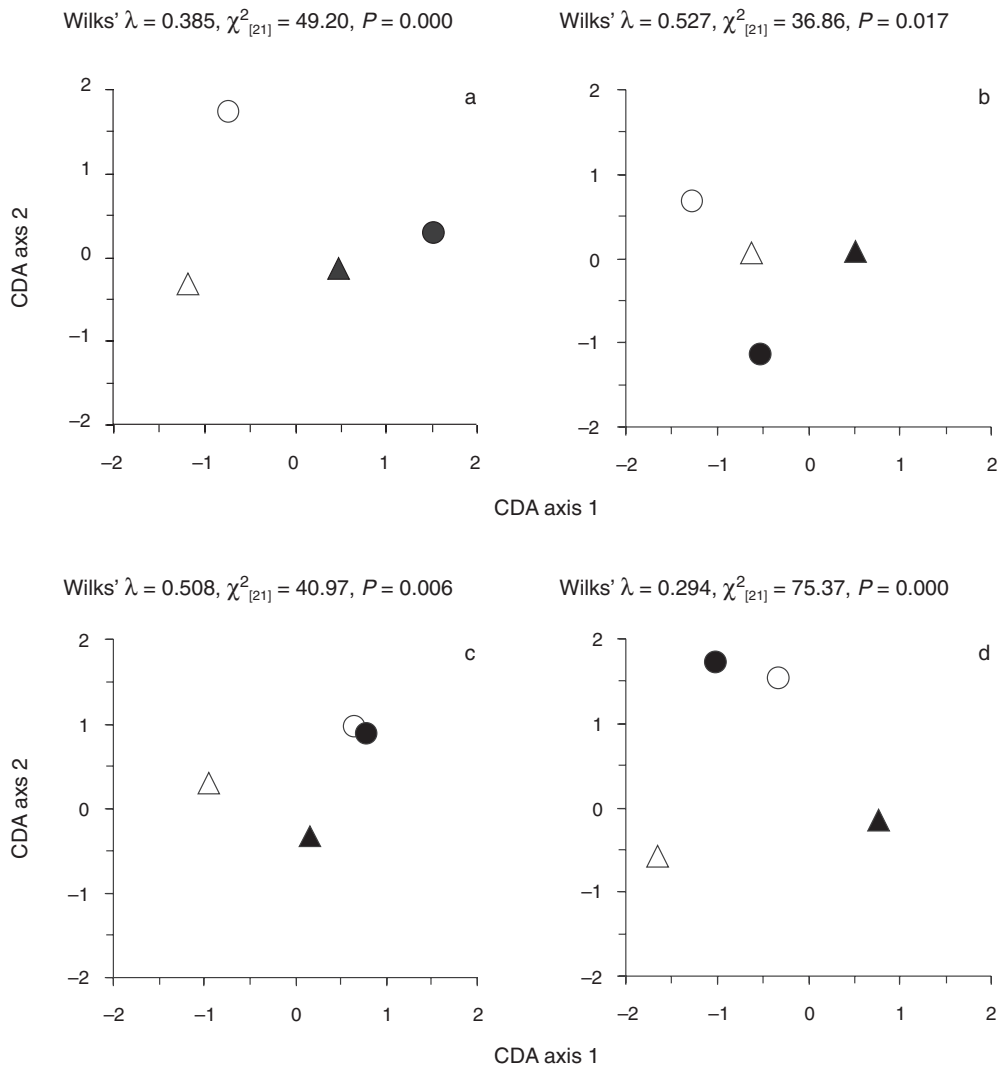


Fig. 5. Canonical discriminant plots showing separation of subspecies *Pararge aegeria tircis* (Southampton early spring cohorts 1.1, 1.111 and 1.441) and *P. a. aegeria* (southwest France cohort 4.1) of both sexes. Location of group centroids with respect to canonical discriminant axes 1 (horizontal) and 2 (vertical) and the significance of their separation, are shown for dorsal forewing (a), ventral forewing (b), dorsal hindwing (c) and ventral hindwing (d). *P. a. tircis*: ▲ = ♂♂, ● = ♀♀; *P. a. aegeria*: △ = ♂♂, ○ = ♀♀.

not readily distinguishable by eye, prove it useful for identifying wing surfaces or features which might warrant visual examination. Canonical analysis, however, estimates the significance of group separations in a manner analogous to the one-way analysis of variance and so is unsuited to the testing of hypotheses involving complex factor effects and interactions. Multi-way analyses of variance on the other hand, can handle several factors and interactions simultaneously and furnish significance levels. For example, the apparently simple sex difference in table 4 is revealed in table 3 to be due to an interaction with sample. However, the possible non-linear relationship between the seven moment invariants (Rohlf, 1990), may render the results of all linear multivariate analyses harder to interpret.

Since the separate-surface and entire-butterfly analyses utilize the same raw data, they would be expected to lead to similar conclusions. Comparison of table 3 and appendix 4b, however, reveals a failure of the MANOVA in the entire-butterfly analysis to detect the two-way interaction effects of sample detected using the separate-surface approach, and a poorer detection by individual moments of their surface specificities. This will have arisen because, unlike the separate-surface approach which enables damaged surfaces to be selectively excluded, the selection criteria necessarily also exclude some usable surfaces thereby losing information. This would account for the poorer detection here of surface-specific effects in individual moments and of sample differences in the MANOVA.

Specimen damage is commonly incurred through natural wear in the field, in the course of breeding programmes, and during preservation. The test photographs showed that simulated wing rubbing and damage obscured some factor effects and damage introduced false effects. In the analyses

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using butterfly specimens, damage again acted as noise, especially when including severely damaged surfaces as in the 149 individual analysis (appendix 4c). It is therefore important that damaged surfaces be excluded from analyses of small samples or where the magnitude of the pattern variation is likely to be small. However, the ability of the moment invariants to discriminate patterns in the presence of some damage contributes to their versatility.

Moment invariants have the advantage over manual methods of utilizing all the available information from the image and being independent of object orientation. They thus provide operational summaries of shape and pattern properties which formerly could only be characterized in terms of arbitrarily chosen measurements such as length-width ratios or subjective qualities such as 'falcate' or 'spotted'. Table 5 compares the efficacy of visual inspection and of the various approaches to moment invariant analysis used in the present study. On this basis, the use of two-dimensional pattern moment invariant methods for the fully automatic measurement of biological patterns such as insect wings is recommended.

Alternatives to the use of moment invariants

Alternatives for describing outline shapes include fractal geometry (Neil & Curtis, 1997) and higher order spectra (Chandran *et al*., 1997). A landmark-based wing pattern method is being explored as a practical alternative to molecular discrimination for distinguishing invading Asian gypsy moths, *Lymantria dispar* (Linnaeus) (Lepidoptera. Lymantriidae), from the indigenous North American population (http://www.bio.umass.edu/biology/kunkel/asian.html).

White and co-workers (White *et al*., 1988; White & Prentice, 1988) have also used Fourier descriptors, whereby repeating curves or patterns are broken down into series of component sine waves or harmonics. This one-dimensional application can be generalized to describe outline shapes using elliptic Fourier coefficients (Kuhl & Giardina, 1982; Rohlf & Archie, 1984), and potentially to two-dimensional patterns. The lower Fourier harmonics, like moment invariants, are understood to summarize the main outline and pattern features, while higher Fourier harmonics (and higher-order moment invariants if developed) encompass finer details of the pattern.

Applications of moment invariants

Moments invariants will be particularly suited to quantifying the properties of individuals within samples or populations where there are many individuals to be measured, and consistent measurement and the avoidance of bias towards particular pattern features are important. The ability to quantify wing features numerically has several potential applications in entomology, including automated identification and population studies. Even wing rubbing, fading or fraying, previously referred to as a nuisance factor, may be of intrinsic interest. For instance, the age of tsetse flies might be determined from the relationship between the pattern quantified and age-related changes such as wing fraying.

Moments are also ideal as a basis for measuring fluctuating asymmetry as an indicator of the developmental stability of populations under isolation or environmental stress. Since fluctuating asymmetry can be calculated from any measurable numerical quantity, it can be applied to moment invariant values even though their relationship to particular properties of the pattern is unknown.

Automated identification systems are being developed to distinguish the species, sex or population of origin of individuals. The use of moment invariants could differentiate morphologically similar taxa. Specific manual pattern features can, of course, still be measured from digitized images (Shreeve, 1985), and used to supplement the use of automatic descriptors. Tang *et al*. (1998) successfully discriminated species of plankton by supplementing their moment vectors with texture descriptors.

Alternatively, moment invariants could be used as an input image standardization stage or 'front-end' for image processing systems, such as the Digital Automated Identification System (DAISY, O'Neill *et al*., 1997; Weeks *et al*., 1997) whose proposed applications include the rapid identification of catches at moth traps. This system uses

Table 5. The efficacy of approaches to wing pattern recognition.

	Single surface			Entire butterfly		
Source:	Visual inspection	Table 3		Appendix 4b		Table 4
Analysis:		Individual moments	MANOVA	Individual moments	MANOVA	Canonical analysis
Interactions and factors						
Sample × sex × treatment	No					***
Sample × sex	No	1	**	2		***
Sample × treatment	No					***
Sex × treatment	Yes[a]					***
Sample	Yes[b]	8	**	15	***	***
Sex	Yes			8		***
Treatment	Yes[a]					**

Columns describe the ability of each analysis to detect significant factors and interactions (number of moment effects significant at $P \leqslant 0.01$). Columns headings denote the unit of analysis (a single surface with 7 moments or the entire butterfly comprising eight surfaces with 56 moments). Significance level conventions as in table 1. [a]specific groups only; [b]specific individuals only.

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images of specimens of known species to train numerical classifiers, which can then be used to determine to which species an unidentified individual is closest. The classifiers do not currently use moments, but it has been suggested that moment invariants could usefully be added to the set of training parameters (Mark O'Neill, personal communication)

Moments are also suited to studies in which pattern is of intrinsic interest. The results may suggest closer examination of patterns to reveal new biological insights. For example, they can be used to study phenotypic trends along environmental gradients, or in response to selective or formative agents, in circumstances where it may be impossible to predict the appropriate traits for study. The ease with which the moment measurements can be adjusted to reflect or ignore object size (White *et al*., 1988), suits them to studies where separate examination of size and pattern trends is desired. For example, *P. aegeria* shows seasonal size and pattern progressions which do not fully correspond (Robertson, 1980). Adjusting for size might facilitate studies of whether pattern formation processes are scaled to the developing morphogenetic field ('morphallaxis') or not ('epimorphosis', Wolpert, 1998).

The first moment m_1 is understood to correspond to the amount of dark area in the wing image, so that larger or darker wings take higher values, while m_2 may be greater for dark areas arranged in a narrow elongated distribution. A better understanding of how individual moments relate to pattern features would enable the use of moment invariants as a tool to identify morphological features that might warrant further attention. Some moment formulations enable patterns to be reconstructed, and the 'average' patterns of different samples to be depicted and viewed, as has been achieved for example with leaf outlines using elliptical Fourier harmonics (White *et al*., 1988).

The ability of the moment invariants to detect pattern differences despite small sample sizes and damaged wing surfaces indicates their potential, as with DAISY, to assist the taxonomic revision of museum series, which may comprise just a few individuals and vary in condition. They could also be computed from digital camera images of live specimens in the field, possibly using a restraining technique such as that of Chesmore & Monkman (1994).

Future developments

One limitation of analysing images captured from unrestrained specimens in the field is that the recorded pattern might suffer distortion to different degrees depending on resting posture and the angle of view, requiring the development of procedures to correct the image or the moment invariants.

Moment computations could be extended to encompass colour information, either by a transformation of colour values to a monochrome scale analogous to the use of colour filters in black-and-white photography, or by using a colour model (such as red-green-blue or hue-saturation-value), in which separate sets of moments are calculated from each colour channel. The choice of transformation or colour model could exploit specific pattern features, thereby enhancing automated species recognition and facilitating developmental studies.

It would be desirable to increase the number of moments in order to capture more usable information. However, rectangular moment invariants in general suffer from discretization effects of the pixel sampling grid and so may not be truly invariant under rotation (Rohlf, in Rohlf & Bookstein, 1990), though this may become less critical with increasing resolution. These issues might be addressed by novel formulations of moment invariants, such as Zernike, pseudo-Zernike and wavelet moment invariants, which are reviewed by Prokop & Reeves (1992) and Liao & Pawluk (1996). A recent introduction can be found in Nixon & Aguado (2002).

Availability of software

The Arbo program was written by White, and runs on PCs and Macintosh computers without any special hardware requirements. Input can be taken from scanner or digital camera image files or from a video camera. The program calculates moment, Fourier and other shape and pattern descriptors; the moment calculations take less than one second per image. The images and data sets used in this study and further details of the program are available at http://www.biodiversity.soton.ac.uk/arbo/.

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Appendix 1

The brightness pattern of an object, represented by a series of pixels, can be described in terms of their Cartesian coordinates x and y and their darkness values $f(x, y)$. In rectangular moment invariant analysis, the *central moments* are statistical descriptions of this pattern:

$$\mu_{pq} = \frac{\sum f(x,y)\ (x-\bar{x})^p (y-\bar{y})^q}{\sum f(x,y)}$$

where p, q are non-negative integers. Central moments do not depend on the location of the object within the image field, but vary as its orientation changes. However, they can be converted to *moment invariants* which are functions of the central moments that do not vary with object orientation. Dudani *et al*. (1977) defined seven moment invariants:

$M_1 = \mu_{20} - \mu_{02}$
$M_2 = a^2 + 4\mu^2_{11}$
$M_3 = b^2 + c^2$
$M_4 = d^2 + e^2$
$M_5 = bf + cg$
$M_6 = a(d^2 - e^2) + 4\mu_{11}de$
$M_7 = cf - bg$

where $a = \mu_{20} - \mu_{02}$, $b = \mu_{30} - 3\mu_{12}$, $c = 3\mu_{21} - \mu_{03}$, $d = \mu_{30} + \mu_{12}$, $e = \mu_{21} + \mu_{03}$, $f = d(d^2 - 3e^2)$, $g = e(3d^2 - e^2)$.

M_1 is a size factor, and M_2 to M_7 can be made independent of object size, using the following scaling (White *et al*., 1988) to serve as shape descriptors m_2 to m_7:

$$r = m_1 = M_1^{1/2}, m_2 = \frac{M_2^{1/4}}{r}, m_3 = \frac{M_3^{1/6}}{r}, m_4 = \frac{M_4^{1/6}}{r}, m_5 = \frac{M_5^{1/12}}{r}, m_6 = \frac{M_6^{1/8}}{r}, m_7 = \frac{M_7^{1/12}}{r}$$

Appendix 2

Means and standard deviations of the moment invariants in the analyses of photographs. Means and standard deviations are shown for the moment invariants m_1 to m_7 calculated from dorsal and ventral images of an undamaged male *Pararge aegeria tircis* butterfly. Values are shown for the three series of replicates: (a) ten identically printed pairs of photographs, (b) eight replicated pairs printed at various densities, and (c) ten types of damage (fig. 1). In series (b), the density level equal to the control replicates was omitted from the means reported to emphasize the differences between the reduced density levels and the control replicates. Since moment values were calculated from both the left and right sides, the number of values in each cell of the table is twice the number of replicated pairs.

Group	m_1	m_2	m_3	m_4	m_5	m_6	m_7
(a) 10 control replicates (n = 20)							
Forewing, dorsal	30.3 ± 0.3	0.72 ± 0.01	0.70 ± 0.01	0.41 ± 0.01	0.44 ± 0.04	0.46 ± 0.01	0.43 ± 0.04
ventral	32.1 ± 0.2	0.71 ± 0.03	0.74 ± 0.01	0.49 ± 0.02	0.50 ± 0.04	0.44 ± 0.06	0.54 ± 0.01
Hindwing, dorsal	28.0 ± 0.1	0.51 ± 0.01	0.65 ± 0.01	0.37 ± 0.02	0.42 ± 0.02	0.38 ± 0.02	0.39 ± 0.04
ventral	31.3 ± 0.3	0.57 ± 0.01	0.67 ± 0.01	0.51 ± 0.02	0.54 ± 0.02	0.53 ± 0.02	0.50 ± 0.04
(b) 7 density levels (n = 14)							
Forewing, dorsal	28.3 ± 4.2	0.65 ± 0.15	0.75 ± 0.06	0.50 ± 0.09	0.53 ± 0.09	0.50 ± 0.06	0.53 ± 0.06
ventral	29.3 ± 3.3	0.65 ± 0.10	0.80 ± 0.05	0.62 ± 0.07	0.65 ± 0.08	0.59 ± 0.08	0.61 ± 0.05
Hindwing, dorsal	26.0 ± 3.5	0.53 ± 0.10	0.78 ± 0.15	0.57 ± 0.23	0.59 ± 0.22	0.51 ± 0.14	0.53 ± 0.13
ventral	28.6 ± 4.5	0.60 ± 0.06	0.78 ± 0.12	0.73 ± 0.13	0.73 ± 0.12	0.68 ± 0.09	0.65 ± 0.09
(c) 10 damage types (n = 20)							
Forewing, dorsal	30.4 ± 1.6	0.71 ± 0.06	0.72 ± 0.08	0.48 ± 0.12	0.51 ± 0.11	0.50 ± 0.11	0.50 ± 0.09
ventral	31.9 ± 1.8	0.71 ± 0.06	0.74 ± 0.07	0.49 ± 0.09	0.52 ± 0.10	0.48 ± 0.10	0.51 ± 0.07
Hindwing, dorsal	28.6 ± 1.4	0.52 ± 0.08	0.61 ± 0.10	0.40 ± 0.13	0.42 ± 0.11	0.38 ± 0.10	0.41 ± 0.12
ventral	31.7 ± 1.6	0.58 ± 0.05	0.63 ± 0.09	0.48 ± 0.10	0.50 ± 0.09	0.48 ± 0.08	0.48 ± 0.09

Appendix 3

Partitioning of variation in the moment invariant measurements. The sums of squares for each main factor in the three four-way analyses (a, b and c, table 1) are expressed as a proportion of the total; for ease of interpretation the sums of squares for interactions have arbitrarily been divided equally among their respective component factors. For each of the seven moments, and for all seven moments combined, this shows the proportion of the variation observed which is attributable to each factor.

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Source of variation	m_1	m_2	m_3	m_4	m_5	m_6	m_7	All seven moments
(a) 10 control replicates								
Wing	0.28	0.94	0.74	0.06	0.10	0.28	0.15	0.36
Aspect	0.71	0.04	0.20	0.90	0.74	0.56	0.71	0.55
Replicate	0.01	0.00	0.02	0.02	0.06	0.08	0.07	0.04
Side	0.01	0.01	0.05	0.02	0.10	0.08	0.07	0.05
(b) 8 density levels								
Wing	0.06	0.31	0.10	0.13	0.11	0.10	0.08	0.12
Aspect	0.11	0.16	0.25	0.27	0.30	0.38	0.33	0.26
Density level	0.79	0.46	0.58	0.56	0.56	0.48	0.53	0.56
Side	0.04	0.07	0.07	0.04	0.04	0.04	0.06	0.05
(c) 10 damage types								
Wing	0.12	0.75	0.50	0.24	0.26	0.24	0.28	0.34
Aspect	0.35	0.04	0.02	0.12	0.10	0.13	0.12	0.13
Damage type	0.52	0.20	0.47	0.60	0.59	0.59	0.56	0.50
Side	0.01	0.01	0.01	0.04	0.04	0.04	0.04	0.03

Appendix 4

Differences and interactions among the samples, sexes and pupal treatments, detected in the entire-butterfly univariate and multivariate analyses of variance at three levels of acceptable damage and value ranges (a–c), showing the number of individual moment variables significant at $P \leqslant 0.01$. Further columns identify moments from the eight wing surfaces: the subscripts (1 to 7) of moments significant at $P \leqslant 0.001$ or (in parentheses) $P \leqslant 0.01$ are listed. The column labelled 'MANOVA' shows the level of significance using the complete set of 56 moments from all eight wing surfaces. Significance level conventions as in table 1.

Interactions and factors			Dorsal				Ventral				MANOVA	
		No. of significant	Forewing		Hindwing		Forewing		Hindwing			
	d.f.	moments	Left	Right	Left	Right	Left	Right	Left	Right	Wilks' lambda	Probability
(a) Worst condition $\leqslant$ 2.0, $m1 > 5.0$ (118 individuals)												
Sample × sex × treatment	2, 80	2	2				2				0.0655	0.384
Sample × sex	7, 80	1				2					0.0012	0.999
Sample × treatment	7, 80	0									0.0001	0.192
Sex × treatment	2, 80	0									0.0767	0.548
Sample	16, 80	10	24	2(45)	(6)		(2)	2	(56)		0.0000	0.005**
Sex	1, 80	3	(3)	(13)							0.2115	0.201
Treatment	2, 80	1	2								0.0742	0.513
(b) Worst condition $\leqslant$ 2.0 and mean condition $\leqslant$ 1.5, forewing $m1 > 17.0$, hindwing $m1 > 15.0$ (90 individuals)												
Sample × sex × treatment	1, 58	0									0.0097	0.092
Sample × sex	5, 58	2				2				(2)	0.0000	0.349
Sample × treatment	4, 58	0									0.0000	0.053
Sex × treatment	2, 58	0									0.0002	0.057
Sample	16, 58	15	(1)345	347 (256)	(1)	(1)		(2)	(1)	1	0.0000	0.000***
Sex	1, 58	8	(1)3	(1)3		(1)	(1)	(1)		(1)	0.0425	0.516
Treatment	2, 58	0									0.0003	0.084
(c) Mean condition $\leqslant$ 2.0, forewing $m1 > 17.0$, hindwing $m1 > 15.0$ (149 individuals)												
Sample × sex × treatment	5, 106	0									0.0571	0.899
Sample × sex	8, 106	1				(2)					0.0007	0.865
Sample × treatment	8, 106	0									0.0005	0.137
Sex × treatment	2, 106	0									0.2918	0.978
Sample	16, 106	1		(5)							0.0000	0.013*
Sex	1, 106	2				(2)			(2)		0.5689	0.966
Treatment	2, 106	1	(2)								0.1590	0.204

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NEW BOOK INFORMATION

From CABI PUBLISHING

Quality Control and Production of Biological Control Agents

Theory and Testing Procedures

Edited by J C van Lenteren, Department of Entomology, Wageningen University, The Netherlands

ISBN	0 85199 688 4
June 2003	352 pages
Hardback	£65.00 (US$120.00)

Readership

Entomology, ecology, crop protection and pest management

Description

The use of biological control agents has been increasing worldwide and there are now many companies mass-producing such organisms, particularly for the control of insect pests. However, there is a great need for quality control in the production and use of these natural enemies, which include insect parasitoids and predators, fungi and viruses. This book has been written by leading scientists from Europe and North America to provide both background theory and practical guidance on this subject.

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NEW BOOK INFORMATION
From
CABI PUBLISHING

Biological Control in the Tropics

Edited by Loke Wai Hong, CAB International South-East Asia Regional Centre, Malaysia, et al

ISBN	0 85199 462 8
January 2000	155 pages
Paperback	£27.50 (US$50.00)

Readership
Research workers in pest management and tropical agriculture.

Description
Biological control has been central to many recent successful integrated pest management programmes. It is also the natural choice where protection of the environment is particularly important. This book reviews current knowledge of biocontrol programmes in the tropics, particularly in developing countries. It is based on papers presented at a symposium held in Malaysia in March 1999. Contributors include authorities from Europe, North America and Australia, as well as from South-East Asia.

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