TECHNICAL NOTE

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The Forensic Application of Allozyme Electrophoresis to the Identification of Blowfly Larvae (Diptera: Calliphoridae) in Southern Australia*

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ABSTRACT: Most known carrion-breeding species of blowflies in southern Australia are of the genus *Calliphora.* The morphological similarity of the immatures of these species means that correctly identifying them poses a challenge for forensic entomologists. This study investigates the potential of allozyme analysis to assist with this task. Molecular profiles of third-instar larvae and adults representing four of these carrion-breeding species, *Calliphora stygia, C. dubia, C. hilli hilli,* and *C. vicina,* were compared at 42 allozyme loci. The two life stages were found to display almost identical allozyme profiles in each species (93% of loci were expressed in both life history stages), enabling the reliable identification of larvae in these four species. Integration of these results with data from a previous study indicates that allozyme analysis would also be suitable for rapid, species-level identification of the larvae of six other carrion-breeding *Calliphora* species occurring in southern Australia. This is the first report of the application of allozyme data to the identification of forensically important blowflies.

KEYWORDS: forensic science, forensic entomology, larvae, blowflies, *Calliphora,* Calliphoridae, allozyme electrophoresis, identification, Australia

Carrion-breeding blowflies (Diptera: Calliphoridae) are well known for their valuable role in the investigation of crimes. They can be particularly useful in cases of murder or suspicious death by helping to provide an estimate of time since death (1,2). In such cases it is important for forensic entomologists to identify the blowflies involved, most often present as larvae, as well as to determine their thermal history (3). Correct identification is particularly important because rates of development under identical temperature regimes can vary markedly between species.

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Unfortunately, blowfly larvae are even more difficult to identify by morphological examination than are the corresponding adults. This problem arises mainly because the external morphology of blowfly larvae is relatively featureless, with few structures exhibiting the variation that might set species apart (4). Thus, blowfly species are much more similar as immatures than as adults, and the larvae of closely related forms may be almost or quite identical (5–7). Although difficulties in identifying larvae can be overcome by rearing them through to adults, this procedure has several practical drawbacks. Specimens require time to complete their development, thus resulting in a delay before identification is possible. Other problems include contamination of cultures by wild blowflies and destruction of irreplaceable specimens by parasitoid Hymenoptera or by the predacious larvae of certain *Chrysomya* species. As a consequence of the latter two factors, a decision to rear the larvae might leave a forensic entomologist without any adult specimens for identification.

The advent of molecular biology has enabled identification of insects using proteins that are expressed by the DNA within cells or by analysis of the DNA itself (8). Since adult and immature insects share the same genotype, molecular techniques have the potential to be used as a reliable means of identifying the immature stages of forensically important blowflies. Recently, this has been achieved with mitochondrial DNA (9), but to date there have been no reports of it having been done using allozyme analysis.

This paper presents the results of a comparison of the allozyme electrophoretic profiles of adults and larvae of four forensically-important species of blowflies in Australia-*Calliphora stygia* (Fabricius), *Calliphora dubia* (Macquart), *Calliphora hilli hilli* Patton and the introduced *Calliphora vicina* Robineau-Desvoidy. The first three of these species were chosen for this study because each resides within a separate, morphologically distinct species-group (the *C. stygia-, C. augur-,* and *C. hilli*-groups, respectively) (10,11), whose larvae nonetheless are quite similar across species-groups. Importantly however, each species-group contains at least one other species, and the immature stages of the species within each group are difficult or impossible to separate on morphological grounds (unpublished observations). The non-native species *C. vicina* was also included because its larvae are commonly found in corpses in the urban areas of southern Australia. On morphological

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grounds it lies within yet another, separate, group (the *C. vicina* [*erythrocephala*]-group) (10).

The aim of the work presented herein was to show that identification of larvae from any of the *Calliphora* species in the above-mentioned species-groups can be achieved by allozyme electrophoretic comparison with an adult of the same species. The comparative allozyme profiles of adults of nine carrion-breeding species of *Calliphora* native to Australia (including *C. stygia, C. dubia,* and *C. hilli hilli*) are already known from a previous molecular systematic study (11). Together, the two studies demonstrate the utility of this molecular technique for larval identification in this systematically biodiverse region of southern Australia.

Materials and Methods

Collection of Specimens

Adults and larvae were obtained from laboratory cultures established by rearing eggs or first instar larvae oviposited by females caught in Adelaide, South Australia. Cultures were maintained in cages at 25 ± 1°C under a 12h:12h light:dark regime and were provided with water, sugar, and lamb's liver as a medium for oviposition. Larvae were allowed to pupate in wheat chaff beneath the rearing dishes. Puparia were then transferred to fresh cages for the emergence of adults.

Individual adults and third instar larvae were placed in Eppendorf tubes, labeled according to their identity, frozen alive in liquid nitrogen and stored at −80°C to await allozyme analysis.

Allozyme Analyses

Whole specimens were homogenized using a sonicator in an equal volume of cold homogenizing solution (12), and centrifuged at 10 000 g for 10 min. The supernatants were then stored at −20°C as separate 5 to 10 μL portions inside glass capillary tubes until required for electrophoresis.

Allozyme electrophoresis was performed on cellulose acetate gels ("Cellogel") according to the principals and protocols outlined in (12) and in Table 1. Adults and larvae of all four species were analyzed together for each of the enzyme systems detailed in Table 1, these being the systems giving sufficient resolution and activity to allow the assignment of genotypes.

Since the comparative allozyme profiles of *C. stygia, C. dubia, C. hilli hilli,* and five other species from the *C. stygia-, C. augur-,* and *C. hilli*-groups were already known from the earlier molecular study (11), it was not necessary to repeat either the full range of taxa nor the large sample sizes per taxon. Only small sample sizes are required to demonstrate that a genetic marker already documented in adults (e.g., *PepD-2* - diagnostic for taxa within the *C. stygia*- and *C. augur*-groups) is both expressed and genetically-interpretable in a larva of the same species. Sample sizes

TABLE 1—*Summary of the electrophoretic methods employed for each allozyme marker.*

Enzyme Abbrev.	Common Name	E.C. No.	Marker	Buffer*	Run Time (min)	Stain†
ACON	aconitate hydratase	4.2.1.3	*Acon-1, Acon-2, Acon-3*	all on B	120	1
ACP	acid phosphatase	3.1.3.2	*Acp*	D	140	1 - method A
ACYC	acyclase	3.5.1.14	*Acyc*	C	165	2
ADH	alcohol dehydrogenase	1.1.1.1	*Adh*	C	135	1
AK	adenylate kinase	2.7.4.3	*Ak*	C	140	1
ALD	fructose-biphosphate aldolase	4.1.2.13	*Ald*	B	150	1 - method A
ARGK	arginine kinase	2.7.3.3	*Argk*	B	120	3 - method 2
DIA	diaphorase	1.6.99	*Dia*	B	120	1 - no MTT
ENOL	enolase	4.2.1.11	*Enol*	A	120	1
EST	esterase	3.1.1.?	*Est-1, Est-2*	C	110	1 - method A
FUM	fumarate hydratase	4.2.1.2	*Fum*	B	140	1
GDA	guanine deaminase	3.5.4.3	*Gda*	B	140	1
GLDH	glucose dehydrogenase	1.1.1.47	*Gldh*	B	150	1
GOT	aspartate aminotransferase	2.6.1.1	*Got-1, Got-2*	B	150	1
GP	non-enzymatic general proteins	—	*Gp*	D	150	1
GPI	glucose-phosphate isomerase	5.3.1.9	*Gpi*	B	140	1
HEX	hexosaminidase	3.2.1.30	*Hex*	C	130	3 - method 1
HK	hexokinase	2.7.1.1	*Hk-1, Hk-2*	D	165	1
IDH	isocitrate dehydrogenase	1.1.1.42	*Idh*	A	125	1
MDH	malate dehydrogenase	1.1.1.37	*Mdh-1, Mdh-2*	B	140	1
ME	malic enzyme	1.1.1.40	*Me-1*	B	85	1
			Me-2	D	13	1
MPI	mannose-phosphate isomerase	5.3.1.8	*Mpi*	B	155	1
NDPK	nucleoside diphosphate kinase	2.7.4.6	*Ndpk*	B	140	4
PEPA	dipeptidase (val-leu)	3.4.13	*PepA*	C	140	1
PEPB	tripeptide aminopeptidase (leu-gly-gly)	3.4.11	*PepB*	C	140	1
PEPD	proline dipeptidase (phe-pro)	3.4.13	*PepD-1, PepD-2*	C	150	1
PEPS	dipeptidase (lys-leu)	3.4.13	*PepS*	C	150	1 - PEPC stain
PGAM	phosphoglycerate mutase	4.2.4.1	*Pgam*	B	120	1
PGM	phosphoglucomutase	5.4.2.2	*Pgm*	C	150	1
PK	pyruvate kinase	2.7.1.40	*Pk-1, Pk-2*	C	110	1
SORDH	sorbitol dehydrogenase	1.1.1.14	*Sordh*	C	150	1
TPI	triose-phosphate isomerase	5.3.1.1	*Tpi-1, Tpi-2*	B	140	1

* The buffer codes refer to those in (12.) † Codes for stain recipes: 1 - (12); 2 - (13); 3 - (14), but wihout the agarose and scaled down to 2 mL final volume; 4 - as for AK stain in (12) with the addition of 6 mg GTP.

were as follows: *C. stygia* (1 adult, 1 larva); *C. dubia* (1 adult, 5 larvae); *C. hilli hilli* (3 adults, 5 larvae); and *C. vicina* (1 adult, 5 larvae).

Results

Of the 42 allozyme markers examined (Table 1), eight were found to be invariant (*Ak, Argk, Fum, Mdh-1, Pgam, Pk-1, Pk-2,* and *Tpi-1*). Thirty-nine of the 42 loci were expressed and could be typed in both adults and larvae for each of the four species. Of the remaining three loci, *Gp* was generally expressed in larvae but not in adults, whereas *Gldh* and *Hk-2* were generally evident in adults but not in larvae. However, none of these three loci is important diagnostically for species determination within any of the species-groups of native blowflies (11).

Allozyme genotypes could readily be assigned to both larval and adult blowflies at each of the 39 loci expressed in both life history stages, regardless of whether the locus was invariant or polymorphic. Given that all of the nine species in the previous allozyme study are diagnosable from their allozyme profiles (11), it is evident therefore that the larvae of any of the other five species in the *C. stygia, C. augur,* and *C. hilli* species-groups can be reliably identified by electrophoretic comparison with an adult of the same species. Although not dealt with here, larvae of the sole member of a fourth group included in the earlier study, *C. maritima* Norris, would clearly also be recognizable electrophoretically. The following eight loci, used collectively, would enable the recognition of an unidentified larva of any of the nine native *Calliphora* species, as well as the nonnative *C. vicina: Acon-1, Dia, Enol, Gda, Me-1, Me-2, Mpi, PepD-2.*

Six of the loci used here were not employed in the previous molecular study (*Adh, Ak, Ald, Gp, Pk-1,* and *Pk-2*). It should be noted that their suitability for discriminating between the species within the *C. stygia-, C. augur-,* and *C. hilli*-groups is therefore as yet unknown.

The results further show that larvae of *C. vicina* have an allozyme profile that is distinctive and easily recognized by comparison with the profiles of the other *Calliphora* species. *Calliphora vicina* displayed pairwise fixed differences from the three native species at between 46 to 64% of loci examined. This level of differentiation is greater than that distinguishing *C. dubia* from *C. hilli hilli* (33% fixed differences), but less than that between the highly divergent *C. stygia* and the other two native species (70 or 73% fixed differences). Thus the representatives of these four species-groups are all diagnosable from one another at multiple allozyme loci (a minimum of 14 loci).

Discussion

The results demonstrate for the first time the practical application of diagnostic allozyme markers, selected from the analysis of adult blowflies, for the identification of their immatures. Over 90% of the markers expressed by adult flies in this study were also expressed in larvae, including all of those shown to be diagnostic in the adults. Other workers on arthropods have also had success using this technique for the identification of larvae, e.g., those of mosquitoes (15), fruit flies (16), pyralid moths (17), chrysomelid beetles (18), and ticks (19), and its usefulness for this purpose in zoology in general has also been outlined (20).

The morphological differences between Australian carrion-breeding blowflies are subtle, and as already implied, this is especially the case in the immature stages. Within the genus *Calliphora* there are three pairs of forensically important sympatric species in southern Australia whose larvae cannot be reliably separated morphologically (unpublished observations). The larvae of other closely related carrion-breeding *Calliphora* species that are as yet unstudied will probably prove equally difficult to identify. This contrasts markedly with the situation in other regions where the forensically important species are similarly well documented. For example, in North America such species lie within over twice as many genera as in Australia and are generally therefore more easily distinguished morphologically (21).

Given the taxonomic difficulty outlined above, allozyme analysis offers great potential as an investigative tool for forensic entomologists working in southern Australia. Of course, the technique is not as procedurally simple as many DNA technologies and presents two main areas of difficulty. First, genetic identification using allozyme markers is a strictly comparative procedure and requires the continual availability of control samples of known identity (12). In this instance, such control samples would consist of frozen whole adults of at least one species within each of the five species-groups of *Calliphora* referred to herein. Such reference material would retain suitable activity for many years when housed at −80°C. Secondly, test larvae must be presented to the forensic laboratory as either live or freshly frozen specimens. This difficulty is unlikely to be a significant issue in southern Australia, where most murder investigations are carried out from a central city base.

Although DNA-based technologies might seem at first glance to be the optimal choice for species-level identification of maggots, the use of allozyme electrophoresis has several notable advantages. First, many specimens can be surveyed simultaneously and very quickly; this is particularly important during the development of diagnostic molecular markers, where numerous specimens from across the geographic range of each species may need to be examined to demonstrate the integrity of each marker. It is also very quick (results are generally obtainable within three hours using the techniques outlined herein), very cost effective, and provides results with a high level of reliability. In contrast, species-specific DNA markers are generally more expensive to develop, often take longer to produce a result, and can be hard to identify in closely related species. Indeed, for mitochondrial DNA, there are both theoretical reasons and observational data to suggest that sister species may continue to display paraphyletic and polyphyletic gene trees for considerable periods of evolutionary time, making it difficult to define diagnostic markers (22–24). Preliminary mtDNA data obtained for *Calliphora* from southern Australian do in fact support this notion. Nonetheless, because DNA analysis is not restricted to viable or freshly frozen specimens, its use may be essential in certain cases.

In forensic investigations misidentification of larvae collected from corpses may lead to errors in the estimation of time since death. The consequences of such errors could be very serious. The technique outlined here provides a ready means of species determination in morphologically difficult taxa without the comparably greater investment in time and money necessary to establish markers using DNA.

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