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ORIGINAL PAPER

Muscle attachment site (MAS) patterns for species determination in European species of *Lucilia* (Diptera: Calliphoridae)

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Abstract Species identification is generally assessed to be more difficult in larval stages than in adult forms. Especially closely related species such as *Lucilia caesar* and *Lucilia illustris* are difficult to identify. The aim of this study was to simplify species determination in *Lucilia* larvae for entomological and forensic purposes. Muscle attachment site (MAS) patterns were previously found to be a good tool for species determination in blowfly larvae. Here, distinctive MAS patterns are presented for European *Lucilia ampullacea*, *L. caesar*, *L. illustris*, *L. richardsi*, *L. sericata*, and *L. silvarum.* A joint pattern for the genus *Lucilia* is provided for a quick classification of a larva to the genus.

Keywords Muscle attachment sites · *Lucilia ampullacea* · *Lucilia caesar* · *Lucilia illustris* · *Lucilia richardsi* · *Lucilia sericata* · *Lucilia silvarum* · Genus pattern · Species determination

Introduction

Flies of the genus *Lucilia* Robineau-Desvoidy, 1830 in the family Calliphoridae are known as green bottle flies because of their brilliant metallic greenish colorations. In Europe, the genus is represented by 11 species (Schumann 1986). Many species of *Lucilia* present strong synanthropic tendencies and high abundance in anthropogenic ecosystems (Greenberg 1973; Rognes 1991). Ubiquity, abundant visiting, and active participation in decomposition of large carrion predestine at least five species of *Lucilia* as potential forensic indicators (Anton et al. 2011; Matuszewski et al. 2008; Matuszewski et al. 2010; Smith 1986): *Lucilia ampullacea*, *Lucilia caesar*, *Lucilia illustris*, *Lucilia sericata*, and *Lucilia silvarum*. The species differ in habitat preferences: They are present in a gradient of environments from dry/open to shadow/forest with a transition of many ecotones and overlapping occurrences of species (Draber-Mońko 1993; Draber-Mońko 1996; Draber-Mońko 2004; Fischer 2000; Fremdt and Amendt 2014; MacLeod and Donnelly 1957; Szpila 1999).

A crucial issue in the application of forensic insects is adequate species identification in the material collected (Amendt et al. 2011). This is generally assessed to be more difficult in larval stages than in adult forms (Byrd and Castner 2009; Smith 1986). Entomological evidence collected at crime scenes or autopsies, however, is usually composed of preimaginal stages (Byrd and Castner 2009; Smith 1986).

Taxonomy of necrophagous blowflies of the Palearctic ecozone was studied thoroughly due to their serious medical and veterinary importance. As a result of these scientific efforts, various keys for identification of European blowflies are available in several publications (e.g., Erzinçlioğlu 1985; Erzinçlioğlu 1996; Lehrer 1972; Rognes 1991; Schumann 1954; Schumann 1971; Szpila 2010; Szpila 2012). Among them are also descriptions, revisions, and keys useful for species identification of preimaginal stages. Articles with attempts to providing keys for identification of larval instars of European species of *Lucilia* were published by Schumann (1954, 1971), Szpila (2010), Szpila et al. (2013), and Velasquez et al. (2010). European species of *Lucilia* were also included in keys dedicated to other zoogeographical zones

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(Ishijima 1967; Knipling 1939; Liu and Greenberg 1989). Characters used in traditional keys are details of the cephaloskeleton, the distribution of spines on particular segments of the larval body, the position of papillae on the anal division, and details of posterior spiracles. Recently, the taxonomic value of some of these characters was critically revised especially in the context of identifying sister species like *L. caesar* and *L. illustris* (Szpila et al. 2013).

An innovative solution for further development of morphological methods in species identification of blowfly larvae is an implementation of muscle attachment site (MAS) patterns (Niederegger et al. 2013; Niederegger and Spiess 2012). MAS is located on the inside of the cuticle in blowfly larvae and presents themselves in a species-specific arrangement on every segment. Visibility of MAS is best obtained in larvae without protuberances of the cuticle such as are present in larvae of *Chrysomya albiceps* or *Chrysomya rufifacies* (Smith 1986). The MAS method can be applied for all larval stages, as the patterns are constant throughout the development of the larvae and change only in size during growth (Niederegger et al. 2013). The present study investigated MAS pattern characteristics for six species of the genus *Lucilia*: *L. silvarum*, *L. illustris*, *L. richardsi*, *L. ampullacea*, *L. sericata*, and *L. caesar* and introduces a basic pattern for the genus *Lucilia*.

The aim of this study was to simplify species determination in *Lucilia* larvae for entomological and forensic purposes. With the rise of genetic applications in insect determination, it was furthermore a desire to counteract negligence of morphological methods which still remain attractive for practitioners in the field of forensic entomology.

Materials and methods

Animals

Adult females were caught using baits with pig or chicken liver located in different habitats (stream banks, forests, rural habitats, and indoors) in Poland for oviposition. The keys of Rognes (1991) and Draber-Mońko (2004) were used for identification. All females were labeled and are available as voucher specimens in the insect collection of the Chair of Ecology and Biogeography, Faculty of Biology and Environmental Protection, Nicolaus Copernicus University in Toruń, Poland.

Resulting larvae were reared to the third instar, killed by dousing with boiling water, and stored in 70 % ethanol. For *L. illustris* and *L. caesar*, several larvae were bred to adult to obtain males for identification confirmation.

Preparation

The larvae were measured to the nearest 0.1 mm using a dissecting microscope (Zeiss Stemi 2000C) with digital camera (Zeiss AxioCam ICc1) and measuring software (AxioVision). All preparation and evaluation steps leading to the condensed patterns were performed as given in our previous publications (Niederegger et al. 2013; Niederegger and Spiess 2012).

Data evaluation

Due to the fact that more closely related species were analyzed than in our previous studies (Niederegger et al. 2013; Niederegger and Spiess 2012), all rows in segments 2–4 were

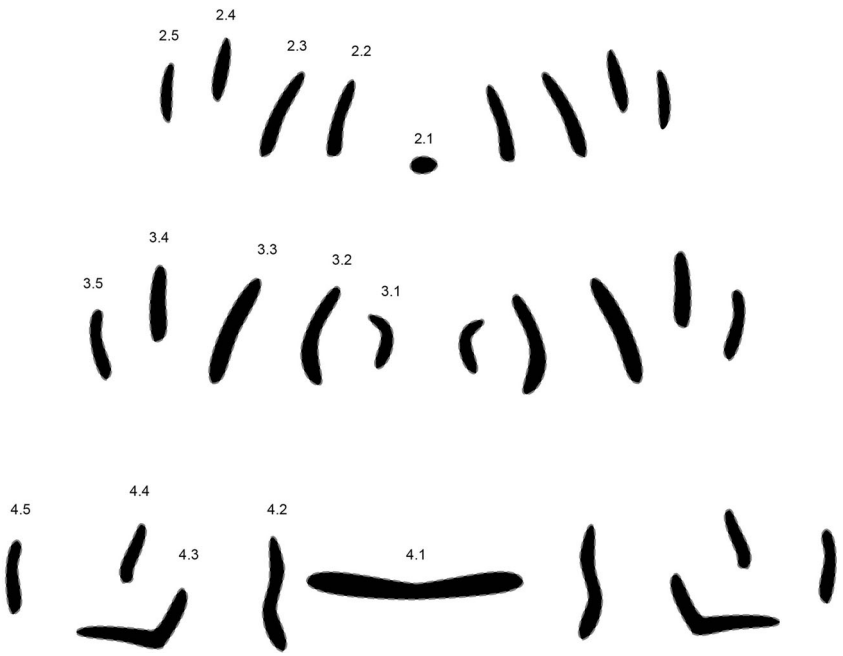

Fig. 1 Genus pattern for *Lucilia* composed of 76 individual MAS patterns of *Lucilia ampullacea*, *L. caesar*, *L. illustris*, *L. richardsi*, *L. sericata*, and *L. silvarum*. *Rows* are labeled according to their location on and affiliation with a segment (e.g., 2.1=central row in segment 2, 4.5=most distal row in segment 4)

documented, and labeling was altered. Starting at the center, rows were numbered according to the segment and the position within the segment (Fig. 1). What was previously known as row 2A now corresponds to 2.2, 3B=3.1, 3A=3.2, 4B=4.1, and 4A=4.2.

The patterns were evaluated using Inkscape (freeware) and Adobe Photoshop; means and standard deviations were calculated using Microsoft Excel 2010.

Results

The attachment sites of the transversal muscles form distinguishable, bilaterally symmetrical rows in each segment. The segments in all species we analyzed showed an average number of 36 (±8) MAS per hemisegment and an average total of 108 (±14) MAS for all three hemisegments (Table 1). The total number of MAS does not correlate with the size of the larvae (Table 2) as was already found in comparisons of less closely related blowfly species (Niederegger and Spiess 2012). Due to the similarities in MAS numbers and positions, we decided to pool all patterns to generate a basic pattern for the genus *Lucilia* (Fig. 1).

Genus pattern (n=76) (Fig. 1)

The genus pattern shows a small row (2.1) in the lower center of segment 2 which is bordered by two almost straight vertical rows (2.2) with slight bends in their middles. More distally located are two vertical rows (2.3) with an angle of about 25° to the midline. The most distal rows 2.4 and 2.5 are about half as long as 2.2 and 2.3; 2.4 is located in the top part of the segment and has an angle of about 10° to the midline whereas 2.5 is lower and almost parallel to the midline.

The center of segment tree is characterized by two small curved rows (3.1) pointing their convex parts at each other and two longer curved rows (3.2) opposing the smaller ones and pointing their concave parts at each other. Rows 3.3–3.5 are slightly larger but very similar to rows 2.3–2.5.

The most conspicuous feature of segment 4 is the horizontal central row (4.1) in which, however, the MAS was often merged to a degree where no individual dots were definable. This row was therefore generally excluded from our analysis. The next rows (4.2) are bent into mirrored S-shapes. The 4.3 is an L-shape with a short row 4.4 on top of it. The most distal row 4.5 again is very similar to the most distal rows in segments 2 and 3.

For the comparison of the genus pattern to individual species patterns, we defined three grades of differences:

- Grade 1: differences in the shape of the rows compared to the genus pattern.
- Grade 2: disruptions and breaks in the pattern.

Table 1 Average numbers of MAS per row structure for six *Lucilia* species and the genus (±STD)

Row	2.1	2.2	2.3	2.4	2.5	3.1	3.2	3.3.	3.4	3.5	4.2	4.3	4.4	4.5
Genus	3 (±1.2)	10 (±1.5)	9 (±1.7)	7 (±1.7)	6 (±1.3)	6 (±1.3)	11 (±1.7)	11 (±1.5)	8 (±1.5)	7 (±1.2)	12 (±1.5)	10 (±2.3)	6 (±1.5)	7 (±2.0)
L. silvarum	2 (±1.7)	9 (±1.3)	9 (±1.6)	6 (±1.9)	6 (±0.9)	5 (±1.4)	12 (±1.8)	11 (±1.3)	6 (±2.4)	7 (±1.3)	11 (±1.2)	11 (±2.1)	5 (±2.2)	6 (±1.3)
L. illustris	4 (±1.0)	10 (±1.3)	10 (±1.1)	7 (±1.4)	6 (±0.7)	5 (±0.9)	11 (±1.8)	11 (±1.7)	8 (±1.9)	7 (±1.3)	11 (±1.7)	12 (±1.6)	7 (±2.1)	7 (±1.1)
L. richardsi	3 (±0.8)	8 (±1.1)	9 (±0.8)	8 (±0.6)	5 (±0.4)	5 (±1.0)	11 (±1.3)	11 (±1.6)	8 (±0.7)	6 (±1.1)	11 (±1.3)	10 (±0.9)	6 (±1.3)	4 (±4.0)
L. ampullacea	3 (±1.4)	11 (±1.3)	12 (±1.3)	8 (±2.0)	6 (±1.7)	6 (±0.8)	13 (±1.7)	11 (±0.9)	8 (±1.0)	7 (±0.8)	12 (±1.0)	12 (±1.3)	7 (±0.8)	8 (±3.7)
L. sericata	4 (±2.1)	9 (±0.6)	9 (±2.2)	8 (±1.4)	4 (±1.0)	6 (±1.0)	10 (±1.5)	10 (±1.3)	8 (±1.0)	6 (±0.6)	10 (±1.5)	11 (±1.7)	6 (±0.8)	7 (±1.3)
L. caesar	1 (±1.6)	10 (±1.5)	8 (±1.5)	6 (±1.4)	6 (±1.5)	7 (±1.5)	11 (±1.4)	11 (±1.7)	8 (±1.2)	7 (±0.8)	12 (±1.4)	8 (±2.1)	6 (±1.5)	9 (±2.6)

Table 2 Average size of larvae compared to the ranges of total MAS numbers per hemisegment

	L. silvarum	*L. illustris*	*L. richardsi*	*L. ampullacea*	*L. sericata*	*L. caesar*
Mean size [mm]	11.15	11.06	12.51	13.39	13.20	12.57
Range no. MAS in 3 hemisegments	93–122	95–128	91–110	108–132	91–119	80–127

- Grade 3: subtle differences (e.g., elongations) or differences in MAS numbers.

Lucilia silvarum (Meigen, 1826) (n=10) (Fig. 2)

The pattern of *L. silvarum* is in accordance with the genus pattern (Fig. 1). All structures are present, and no shape differences (grade 1) or pattern disruptions (grade 2) are apparent. The average numbers of MAS for each row correspond to the average numbers for the genus (Table 1).

Condensed patterns of *L. silvarum* seem to be taking up much less room than indicated by the genus pattern. This is due to the high number of larvae from different species taken into account for the genus pattern and is valid for all of the following species patterns.

Lucilia illustris (Meigen, 1826) (n=16) (Fig. 3)

The pattern of *L. illustris* also corresponds well with the basic pattern for the genus *Lucilia* (Fig. 1). All rows are present, and no shape differences can be detected. Indications for possible pattern disruptions can be found in segment 2 (2.3 in left hemisegment, dotted arrow), but they did not come through for all larvae examined. On the third segment, however, pattern disruptions were found in structures 3.3 (solid arrows) for all larvae and in both hemisegments. *L. illustris* has a comparable average number of MAS for each row (Table 1) as found in the genus pattern.

Lucilia richardsi Collin, 1926 (n=7) (Fig. 4)

In *L. richardsi* pattern disruptions were found in rows 2.3 and 3.3 (arrows) for all larvae and in both hemisegments. The gaps in row 3.3 are wider than those in *L. illustris* (Fig. 3) and also very distinct on both sides in 2.3. These wider gaps, however, did not result in a change of MAS numbers for the rows compared to the genus pattern (Table 1). The very short patterns in rows 4.5 were not taken into account, as the row was not clearly visible in all examined larvae.

Lucilia ampullacea Villeneuve, 1922 (n=10) (Fig. 5)

Pattern disruptions can be found in patterns of *L. ampullacea* in 2.3 and 3.3. (long arrows) and shape elongations (grade 3)

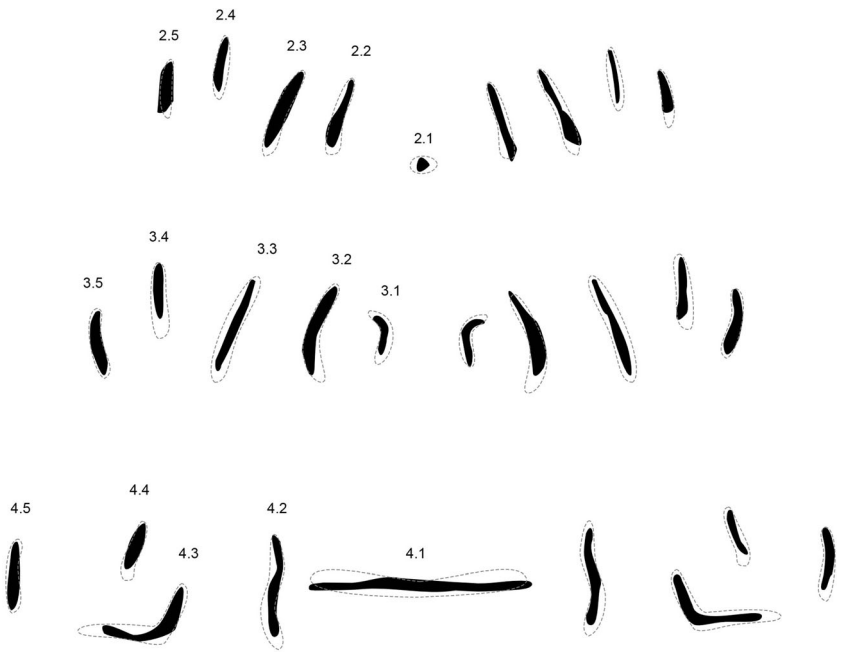

Fig. 2 Condensed MAS pattern for *Lucilia silvarum* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment

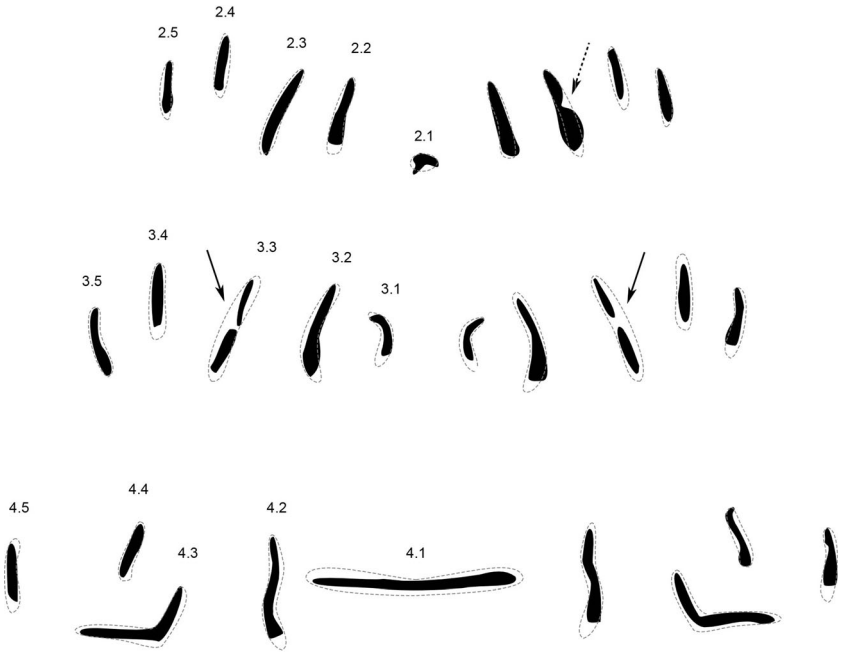

Fig. 3 Condensed MAS pattern for *Lucilia illustris* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern

in 2.2, 2.3, and 3.2 (short arrows). This is also reflected in the average numbers of MAS which is higher than that in the genus pattern (Table 1).

Lucilia sericata (Meigen, 1826) (n=15) (Fig. 6)

Pattern disruptions were found in patterns of *L. sericata* in 2.3 and 3.3 (long arrows). Additionally, shape elongations were found in rows 4.3 (short arrow), although not in the same intensity for all larvae and not always on both sides of segment 4. MAS numbers of rows show no discrepancies compared to the genus pattern (Table 1).

Lucilia caesar (Linnaeus, 1758) (n=18) (Fig. 7)

In *L. caesar*, we found neither pattern disruptions nor elongations, but grade 1 differences in the form of shape change in rows 4.3 (arrows). The typical L-shape could only be found in

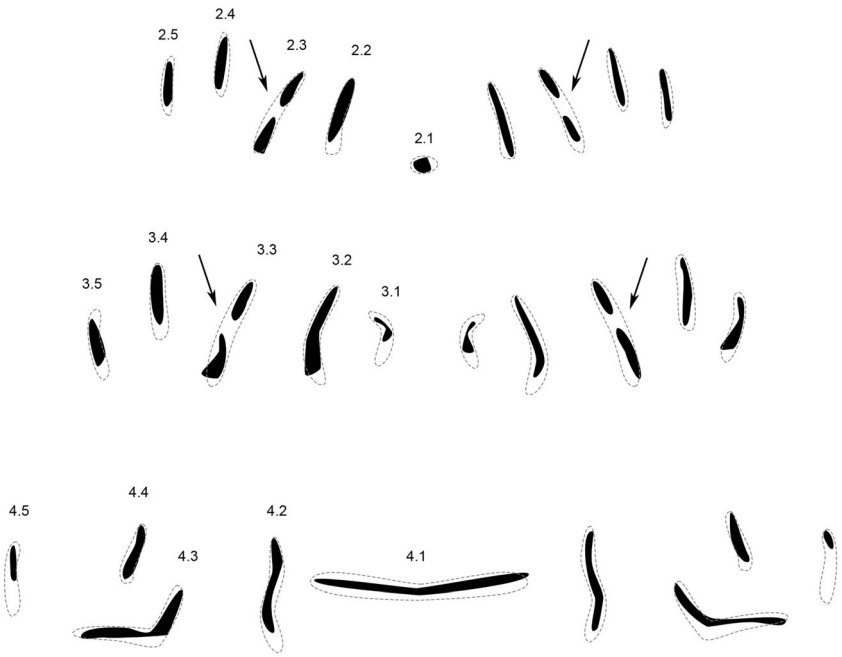

Fig. 4 Condensed MAS pattern for *Lucilia richardsi* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern

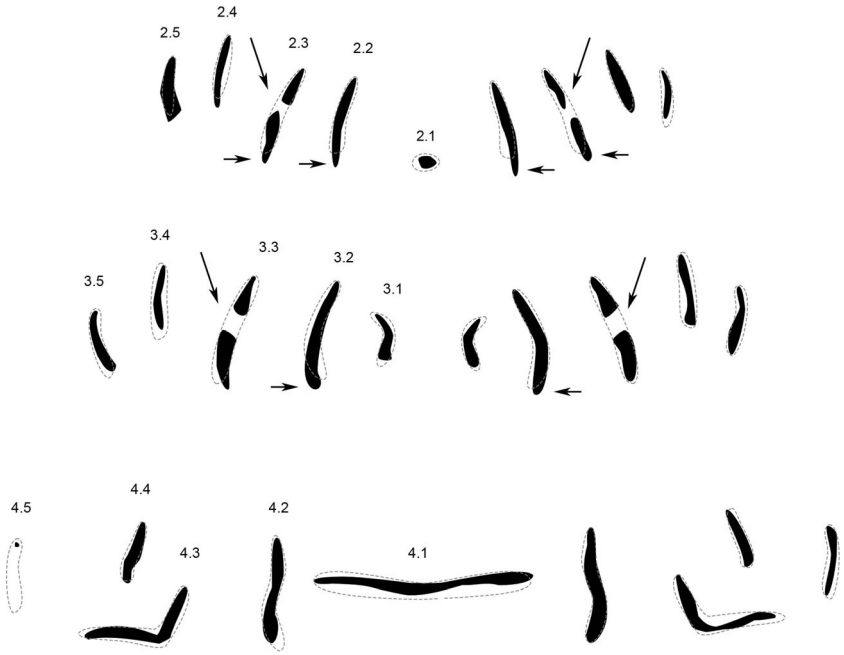

Fig. 5 Condensed MAS pattern for *Lucilia ampullacea* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern

four larvae and only on the right hemisegment. All other larvae were lacking this feature and reduced the L-shape to a straight line with an angle of about 25° to the ventral midline. In five larvae, 4.5 showed a pattern similar to an L-shape opposing the expected shape of 4.3 as a possible compensatory measurement. This, however, did not manifest in the condensed patterns for *L. caesar*. MAS numbers of rows reflect both these differences, as 4.3 in average has fewer and 4.5 has more MAS than the basic pattern (Table 1).

L. caesar vs *L. illustris* (Fig. 8)

Determination is exceptionally difficult between *L. caesar* and *L. illustris* when using traditional morphologic and genetic methods (Rognes 1980; Rognes 1991; Schumann 1971; Sonet et al. 2012; Spence 1954; Szpila 2010). Using the MAS method, however, distinct differences can be found: In *L. illustris* (dotted), pattern disruptions were found in rows 3.3 (long arrows) and L-shaped elongations in 4.3 (short arrows) whereas in *L. caesar*

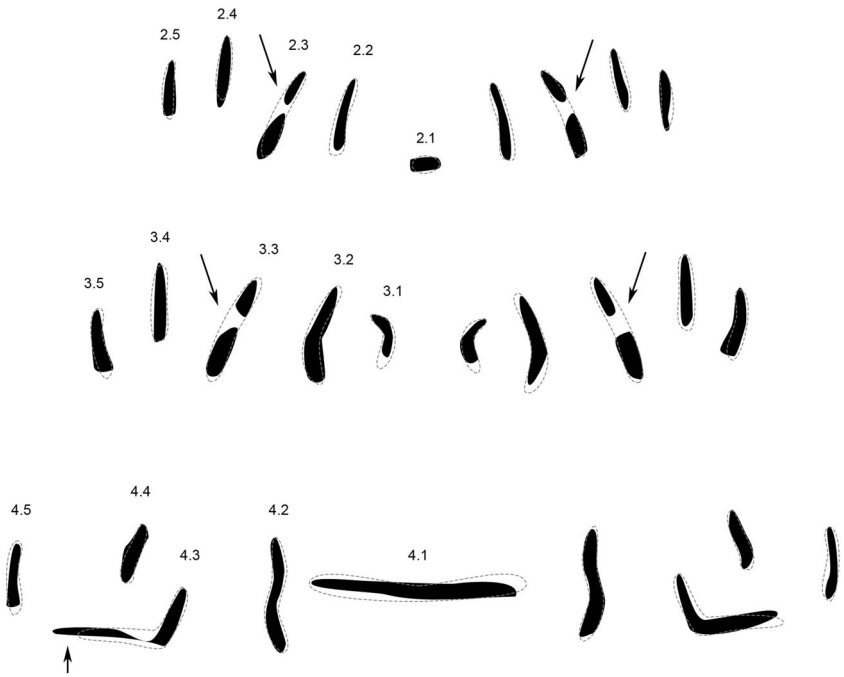

Fig. 6 Condensed MAS pattern for *Lucilia sericata* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern

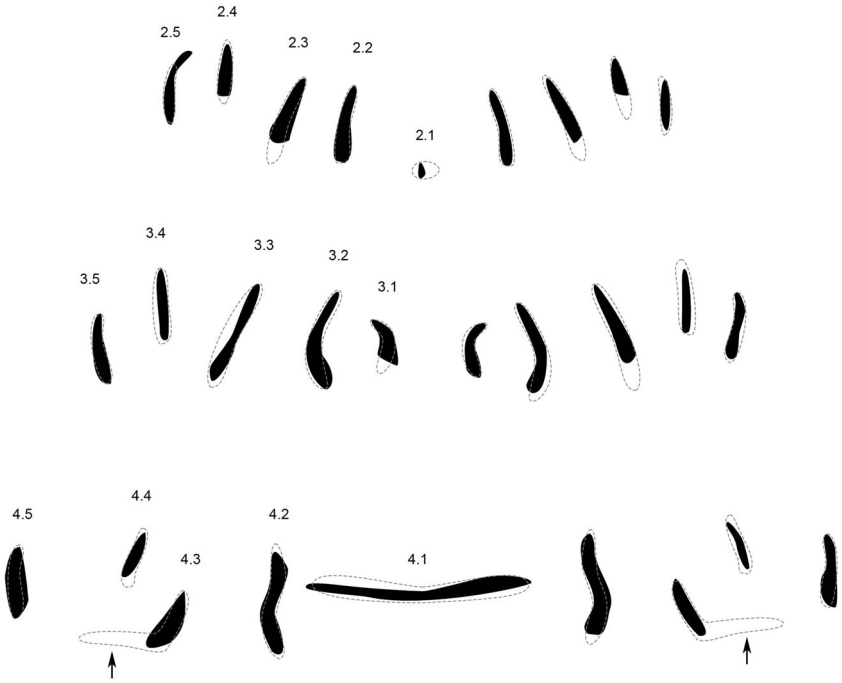

Fig. 7 Condensed MAS pattern for *Lucilia caesar* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern

(solid), neither pattern disruptions nor L-shapes could be detected. MAS numbers differ for 4.3 but not for 3.3 (Table 1).

Discussion

In our previous work, we established the MAS method as promising tool for species determination in blowfly larvae. We challenged our method in this study, and it proved to deliver conclusive results for preimaginal stages of closely related and only recently diverged species (McDonagh and Stevens 2011) of the genus *Lucilia*. As expected, we had to broaden our field of analysis by including more MAS rows but were able to contain it to three segments in order to keep the method simple. The increased number of addressable rows required new labeling which can be continued if more segments should be needed in further investigations. Muscular patterns in *Drosophila*,

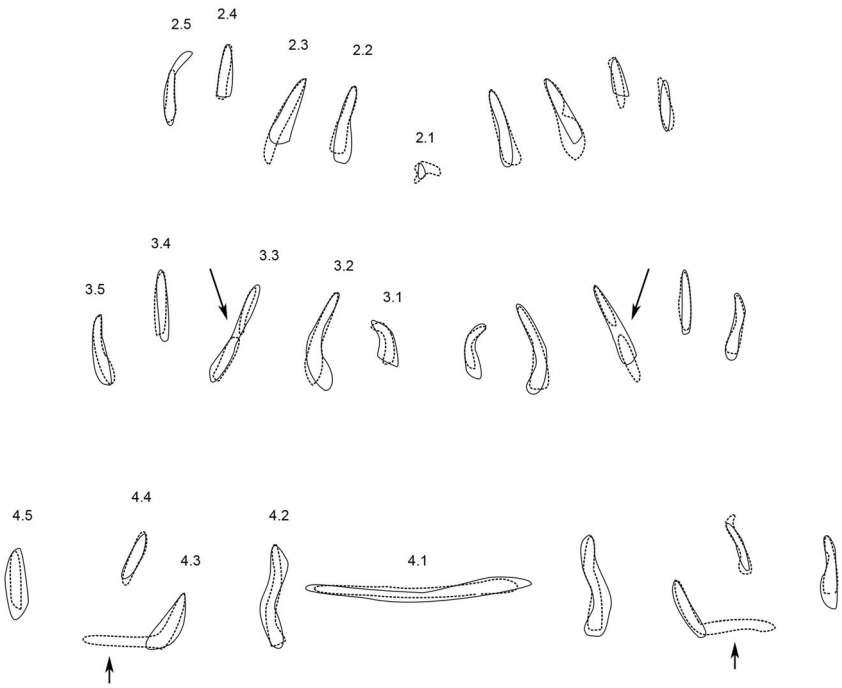

Fig. 8 Outlines of condensed MAS patterns for *Lucilia caesar* (*solid lines*) superimposed with outlines of the condensed muscle attachment site patterns for *Lucilia illustris* (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences between the two species patterns

however, were found to be unique only in the three thoracic and the first and last abdominal segments (Hooper 1986). Similar results were found in *Calliphora vomitoria* (Crossley 1965) and might be valid for all Calliphoridae species.

A joint pattern for *Lucilia* species could be found (Fig. 1) for a quick classification of the larvae to the genus. Species MAS patterns were then compared to the genus pattern. Mostly, grade 2 differences could be found, but also, grade 1 differences were present in at least one species. Grade 3 differences helped confirming results. A number of very small discrepancies to the genus pattern was present in each species. We concentrated on the most apparent differences, however, in order to provide an easy template for quick determinations of individual larvae. For *L. illustris* and *L. caesar*—where species determination of larvae was reported to be difficult (Smith 1986; Szpila 2010; Szpila et al. 2013) or impossible (Schumann 1971) even with genetic methods (Sonet et al. 2012)—we found distinctive features in their respective MAS patterns. Larvae originating from different habitats and different mothers displayed accordant MAS patterns.

Even though the MAS method is simple and quick, results can be improved by paying special attention to careful sampling, collection, and storage of larvae according to standards and guidelines of the European Association for Forensic Entomology (Amendt et al. 2007). The method can be applied to all larval stages. If the larvae are near to pupation, however, the cuticle forms a puparium which includes some chemical changes (Dennell 1958). This might result in fading or disappearance of MAS.

To further establish the method of MAS patterns, more dipteran species should be examined to find more genus and species patterns. Closely related species—such as *L. illustris* and *L. caesar*—from other continents should be analyzed and compared.

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