RESEARCH ARTICLE

Potential application of Gustatory Receptor 1 (CmegGr1) gene as a molecular marker for identification of Chrysomya megacephala (Diptera: Calliphoridae)

Ghazali, S.N.A.1, Emelia, O.1, Hidayatulfathi, O.2, Syamsa, R.A.1*

1Department of Parasitology and Medical Entomology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia
2Biomedical Science Programme, Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
*Corresponding author: syamsarizal.abdullah@ppukm.ukm.edu.my

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ABSTRACT

Chrysomya megacephala larvae can easily be identified using cheap traditional microscopy techniques. Nevertheless, identification using taxonomy keys may be hampered, if the morphological characteristics of the larvae are incomplete, or immature for microscopic identification. To overcome the difficulty of species determination, molecular identification has gained relevance and is applied in forensic investigations. This study aimed to identify a novel target gene, known as the gustatory receptor 1 gene (CmegGr1), which has never been used for identification. The third instar larvae of Ch. megacephala (n = 30) and eight other forensically important fly species were obtained from two sources; rabbit carcasses and the Forensic Entomology Unit collection. Their DNAs were extracted and the CmegGr1 gene was amplified using polymerase chain reaction (PCR). The resulting sequences were subjected to phylogenetic analysis. A 209 bp fragment of the CmegGr1 gene was successfully amplified in 80% (24/30) of Ch. megacephala samples, while all of the non-Ch. megacephala species were not amplified. The phylogenetic analysis revealed that the evolutionary tree of CmegGr1 shares many traits with the 21a gustatory receptors of Calliphora stygia and Lucilia cuprina (Gr21a), which are also classified as necrophagous fly species. The high specificity of species identification was demonstrated in the present study using DNA barcoding, which led to the conclusion that the CmegGr1 gene could serve as an alternative marker for identifying Ch. megacephala.

Keywords: Forensic larvae; gustatory receptor 1 gene; Chrysomya megacephala; DNA identification; phylogenetic study.

INTRODUCTION

Blowflies play an important role in estimating minimum postmortem interval (PMI), because several species are often found in human corpses. In Malaysia, the most prevalent calliphorid species discovered in corpses are Chrysomya megacephala (Fabricius, 1794) (Diptera: Calliphoridae) and Chrysomya rufifacies (Macquart, 1843) (Diptera: Calliphoridae) (Syamsa et al., 2017). The strong association between Ch. megacephala larvae and decaying matter has led to its usefulness in assisting entomologists determine the time of death (Sukontason et al., 2008; Thevan et al., 2010). Its reputation as an early and dominant coloniser of corpses makes it a particularly attractive species for assisting forensic investigation (Kavitha et al., 2012; Syamsa et al., 2017).

The traditional identification of blowfly species in the field is based on morphological techniques that compare the differences in the adults, or matured larvae of forensic fly species across a wide range of features. However, the process of identifying and detecting this species is cumbersome, as the morphologies of the immature larvae are similar to other species. This problem can be overcome by rearing immature larvae until they are fully grown. However, in some cases, the larvae may end up dying, thus, hindering the identification process.

A significant number of studies that apply molecular approaches in identifying these flies has been conducted mainly by comparing known fly DNA sequences (Stojak, 2014). Previous studies have reported the potential of using a “barcoding region” within the mitochondrial DNA, namely, the cytochrome oxidase I (COI) gene, as a universal marker for molecular identification of the forensically important Diptera (Boehme et al., 2012). The COI gene has been established as a useful molecular marker in identifying blowfly species in Africa, Australia, Belgium, England, Germany, Lebanon, and Malaysia (Harvey et al., 2003; Boehme et al., 2010; Tan et al., 2010; Boehme et al., 2012; Jordaens et al., 2013; Shayya et al., 2018). However, the COI region was found to be inefficient in identifying Ch. megacephala, with recent studies demonstrating a possible hybridisation and an incomplete lineage sorting between this species and Chrysomya saffranea (Bigot, 1877) (Badenhorst & Villet, 2018). Therefore, it is crucial to find a new marker gene for this genus to avoid misinterpretations during DNA identification.
The blowfly is naturally attracted to the odour of a decaying corpse and can reach one within minutes to lay eggs (Anderson, 2004; Wells & Stevens, 2008; Mahat & Jayaprakash, 2013; Mona et al., 2019). A gustatory gene in *Ch. megacephala*, which is classified under the chemoreceptor superfamily, is responsible for the sense of taste that corresponds to feeding behaviour, initiation of innate sexual responses, as well as reproductive responses (Sánchez-Gracia et al., 2009). According to Wang et al. (2013), the *Ch. megacephala* gustatory receptor 1 gene (*CmegGr1*) is highly expressed in the antennae and proboscises with maxillary palps in adult flies. As such, this is a promising target that could assist forensic scientists in identifying this species. The present study aimed to determine the potential of using the *CmegGr1* gene as a suitable marker in the identification of *Ch. megacephala* larvae. This study has also compared the availability of this gene in a group of *Ch. megacephala* larvae and several forensically important larvae species.

**MATERIALS AND METHODS**

**Sample collections**
The third instar of *Ch. megacephala* larvae were collected from a simulation of rabbit carcasses at a forensic simulation site at Universiti Kebangsaan Malaysia (UKM), Selangor, Malaysia. The use of rabbit carcasses was approved by the UKM Ethics Committee (Approval No: PARAST/PP/2019/SYAMSA/30-JAN.2019-DEC.-2019-AR-CAT2.). The specimens were collected and directly preserved in 70% of ethanol. The collected samples were stored at 4°C in the Entomology Laboratory, Faculty of Medicine, UKM. All posterior and anterior spiracles were stored as vouchers.

**DNA Extraction**
The genomic DNA was extracted from 30 samples of *Ch. megacephala*, which consisted of 2–5 pooled larvae in each sample. Approximately 25 mg of body tissue samples were used for each extraction and the extracted DNAs were stored at −20°C. The genomic DNA was extracted using the QIAamp® DNA Mini Tissue Kit (Qiagen, Germany) according to the manufacturer’s instructions, with some modifications. After being incubated overnight in an ATL buffer, all samples were ground using sterile 1.5 mL tube plastic pestles. Next, the samples were incubated for 5 min at 56°C. The extracted DNA was then eluted with 200 µL of AE buffer, and eluted again after standing for 5 min in the AE buffer. The purity of the extracted DNA was quantified using a Nanodrop 2000c (Thermo Scientific®, USA) at an absorbance wavelength of A260/A280.

**Polymerase Chain Reaction**
The set of primers was designed using a Primer Explorer software (version 5) using the DNA template from *Ch. megacephala* gustatory receptor 1 (Gr1), with NCBI number of JQ365174.1. The purified DNA was subjected to PCR amplification of CmegGr1 gene following the protocol mentioned in Table 1. The target fragments were amplified with the primer pairs of CmegGr1-F/CmegGr1-R for CmegGr1 DNA fragment size of approximately 209 bp. The PCR mixtures contained ~150 ng DNA template, 1 unit of Taq DNA polymerase (Promega, USA), 1 × PCR reaction buffer (Promega, USA), 1.5 mM MgCl2 (Promega, USA), 200 µM of each dNTP (Promega, USA), 0.4 µM of each forward and reverse primer, and ddH2O to total up the mixture to 50 µL per reaction. The PCR was performed using an Eppendorf Mastercycler Pro Thermal Cycler. The temperature regime used was according to Tan et al. (2010) except for the annealing temperature which was optimised to be at 50°C for 1 min and 30 sec (Table 1). The amplified products were visualised by electrophoresis using 2% of agarose gel at 180 V for 30 min.

**Sequence Analysis**
Samples that showed positive results were sent for sequencing analysis using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) after undergoing purification using the HiYield Plus Gel/PCR Mini Kit (Real Biotech Corp, Taiwan). Gene sequence analysis and database comparisons were performed using the BLAST programme (http://www.ncbi.nlm.nih.gov/blast/). Nucleic acid sequences of nine additional insect species have been retrieved from GenBank, as shown in Table 2. All sequences were aligned and trimmed using the BioEdit Sequence Alignment Editor, version 7.2.6 (Hall, 1999). Samples and database sequences were used to construct a phylogenetic tree to illustrate the relationships of each gene between species trees. The evolutionary tree was inferred using the Maximum-likelihood method with 1000 bootstrap replicates.

**Specificity test**
PCR was performed on the other species of larvae using a similar protocol used for CmegGr1 (Table 1). DNA isolated from *Ch. megacephala* was used as a positive control. The non-*Ch. megacephala* species were selected based on the availability of sources from the laboratory collections, namely, *Chrysomya rufifacies; Chrysomya nigripes* Aubertin, 1932; *Chrysomya villeneuvi* Patton, 1922; *Sarcophaga* spp., *Sarcophaga neculata* Wulp, 1883; *Hypopygiopsis violacea* (Macquart, 1835); *Hemipyrellia ligurriens* (Wiedemann, 1830); and *Musca domestica* Linnaeus, 1758.

**RESULTS**

**Detection of CmegGr1 from larvae**
From the 30 samples of *Ch. megacephala* larvae, 80% (24 samples) were detected with the CmegGr1 gene (Figure 1). The DNA sequence analysis revealed that the extracted samples from the larvae contain 96% to 98% DNAs that are similar to the DNA of the adult CmegGr1 gene (Figure 2A). Figure 2B shows that the phylogenetic tree of CmegGr1 has close characteristics as the 21a gustatory receptors (Gr21a) of *Calliphora stygia* and *Lucilia cuprina*.

**Table 1.** The sequenced primer used in this study and the temperature set for thermocycler

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’-3’</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CmegGr1-F</td>
<td>CACCACTTAAAGATACCTCATCT</td>
<td>Pre-denaturation: 94°C [5 minutes]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denaturation: 94°C [1 minute]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing: 50°C [1 min 30 sec]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension: 72°C [2 minutes]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final extension: 72°C [5 minutes]</td>
</tr>
<tr>
<td>Hold: 4°C [∞]</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
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<tr>
<th>Primer</th>
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<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CmegGr1-B</td>
<td>TACGAGCAAACTTTTGGTAG</td>
<td>Pre-denaturation: 94°C [5 minutes]</td>
</tr>
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<td></td>
<td></td>
<td>Denaturation: 94°C [1 minute]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing: 50°C [1 min 30 sec]</td>
</tr>
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<td></td>
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<td>Extension: 72°C [2 minutes]</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Hold: 4°C [∞]</td>
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</table>

**Table 2.** GenBank ID used for sequence analysis

<table>
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<tr>
<th>GenBank ID</th>
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<tr>
<td>MH750487.1</td>
<td>Drosophila majavensis</td>
<td>United States of America</td>
</tr>
<tr>
<td>MH750458.1</td>
<td>Drosophila arizonae</td>
<td>United States of America</td>
</tr>
<tr>
<td>MH750483.1</td>
<td>Drosophila navojoa</td>
<td>United States of America</td>
</tr>
<tr>
<td>AB042625.2</td>
<td>Drosophila melanogaster</td>
<td>Japan</td>
</tr>
<tr>
<td>KR674136.1</td>
<td>Athetes dissimilis</td>
<td>China</td>
</tr>
<tr>
<td>XM0132411847.1</td>
<td>Stomoxys calcitrans</td>
<td>United States of America</td>
</tr>
<tr>
<td>JQ36177.1</td>
<td>Musca domestica</td>
<td>United States of America</td>
</tr>
<tr>
<td>XM005189934.2</td>
<td>Musca domestica</td>
<td>United States of America</td>
</tr>
<tr>
<td>KJ702098.1</td>
<td>Calliphora stygia</td>
<td>Australia</td>
</tr>
<tr>
<td>XM023443869</td>
<td>Lucilia cuprina</td>
<td>United States of America</td>
</tr>
<tr>
<td>JQ365174.1</td>
<td>Chrysoma megacephala</td>
<td>China</td>
</tr>
</tbody>
</table>
Detection of CmegGr1 in different species of larvae

Figure 3 illustrates the specificity results of CmegGr1 towards Ch. megacephala species only. CmegGr1 was exclusively present in Ch. megacephala samples, but not in the other necrophagous forensic larvae. All samples from Ch. rufifacies, Ch. nigripes, Ch. villeneuvi, Sarcophaga sp., Synthesiomyia nudiseta, Hypopygiopsis violacea, Hemipyrellia ligurriens, and Musca domestica were found to be absent of CmegGr1.

DISCUSSION

In the context of using molecular techniques for identifying necrophagous larvae, it is crucial to choose an appropriate DNA target to facilitate and complement the morphology-based species identification. In general, the COI gene, which consists of ~1588 bp nucleotides, is the most common target amplicon used for molecular identification of the Ch. megacephala (Nelson et al., 2012; Sharma & Singh, 2015). However, previous studies found that the COI gene was not specific for the detection of Ch. megacephala (Kavitha et al., 2012; Badenhorst & Villet, 2018). Although this region is used extensively, the lack of amplification specificity against other carrion-breeding blowflies, such as Chrysomya pinguis (Walker, 1858), Chrysomya bezziana Villeneuve, 1914, Chrysomya pacifica Kurahashi, 1991, and Chrysomya chani Kurahashi, 1979 highlights the shortcoming of this sequence for exclusively identifying Ch. megacephala (Kavitha et al., 2012; Bharti & Singh, 2017; Pedales & Fontanilla, 2018).

Thus, the current study has focused on identifying a potential gene marker, as an alternative for the current use of barcoding markers. A new set of primers targeting the specific amplification of CmegGr1 gene was designed. The PCR results in this study showed a high specificity (100%) of the gene in distinguishing Ch. megacephala apart from other fly species. Hence, the application of CmegGr1
as a specific genetic marker could be used to specifically identify this fly species from a sample containing a large species of forensic flies. Nevertheless, the detection of this gene was noticeably less sensitive (80%) when the conventional PCR method was used, as it originates from nuclear markers, which only have a few copies within the blowfly's genome (Mona et al., 2019). Fortunately, this shortcomings can be circumvented by utilising other methods, such as real-time PCR or microarrays, which are known to possess higher sensitivity.

The phylogenetic analyses revealed that the 209 bp of CmegGr1 gene could differentiate *Ch. megacephala* from other species, as the sequence did not hybridise with the gustatory receptors of other insects. This study also found that the closest molecular characteristics of CmegGr1 were connected to the 21a gustatory receptors of *Calliphora stygia* and *Lucilia cuprina*, namely, CstGr21a and LcupGr21a. The molecular analysis conducted by Leitch et al. (2015) also showed that CmegGr1 has molecular characteristical connections with CstGr21a and the gustatory gene from *Drosophila melanogaster* Meigen, 1830, which is known as DmelGr21a. These receptors were found to be important carbon dioxide receptors, which might function as one of the important receptors for detecting volatile organic compounds for *Ch. megacephala*. Wang et al. (2013) have also determined the function of CmegGr1 gene extracted from adult *Ch. megacephala* as being one of the carbon dioxide receptors. Carbon dioxide is a type of odorant, cuticular hydrocarbon gas, which is highly volatile and important as a long-distance cue for carrion detection (Yang & Shiao, 2012; Dong et al., 2016). Interestingly, carbon dioxide is the main gas produced by a carcass at 80%, followed by hydrogen at 10% at a constant temperature of 25°C (Sakata et al., 1980).

The species-specific characteristics of carbon dioxide receptors in other fly species and the selection of partial CmegGr1 gene as an amplification target in this study have contributed to the high specificity in molecular identification of *Ch. megacephala*. The location of the amplicon was at the N-terminal of the protein and at intracellular (Wang et al., 2013). As the location of the sequences was not at transmembrane, the amino acids can easily adapt to the selective preference of the species. According to Robertson and Kent (2009), some insects were born without carbon dioxide receptors (e.g., honeybee, parasitoid wasp, human louse, pea aphid, water flea, and blacklegged tick), while others have carbon dioxide receptors (e.g., *Anopheles gambiae*, *Aedes aegypti*, and *Culex pipiens*). The behaviour of these insects that are attracted to either humans or corpses was paralleled to the presence of carbon dioxide receptors.

In addition, a study by Sánchez-Gracia et al. (2009) supported that most gustatory receptor families are unique and species-specific based on the evolutionary genome of chemosensory gene families using data from fully sequenced insect genomes from the 12 newly available Drosophila genomes. The sequence alignment of the gustatory receptors of *Ch. megacephala* has shown that the CmegGr1 and CmegGr2 amino acid sequences had different lengths in their N- and C-terminal regions compared to other insect species, thus, providing distinguishing points for *Ch. megacephala* against other insects (Wang et al., 2013). This observation supports the results obtained in this study. Overall, the gustatory gene has shown high specificity for insect identification through molecular detection methods and has the potential as a reliable marker for genetic analysers in the fieldwork.

This study has highlighted the exclusivity of CmegGr1 gene as a new identification marker for *Ch. megacephala*, which can be beneficial in forensic investigations. The uniqueness, high specificity, and expression of the gene at the immature stages of the fly enables it to be further developed as a diagnostic marker to ease the identification of *Ch. megacephala* at forensic sites. This study can provide validation for future studies to reinvestigate similar genes in other species.

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Conflict of Interests

The authors declare that they have no conflict of interests. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

REFERENCES


