Universidade Federal de Pernambuco

Centro de Ciências Biológicas

Laboratório de Imunopatologia Keizo Asami

Programa de Pós-Graduação em Biologia Aplicada à Saúde

DNA HUMANO EXTRAÍDO A PARTIR DE LARVAS DE DÍPTEROS COLETADAS EM CADÁVERES NO INSTITUTO MÉDICO LEGAL DE PERNAMBUCO

TATIANA COSTA DE OLIVEIRA

Recife-PE
2015

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Doutoranda: Tatiana Costa De Oliveira
Orientador: Prof. Dr. Sergio Crovella

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Parecer da comissão organizadora da tese de doutorado de

TATIANA COSTA DE OLIVEIRA

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A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera o candidato **TATIANA COSTA DE OLIVEIRA** como:

APROVADA

Recife, 1º de dezembro de 2015.

_____

Prof. Dr. Sergio Crovella
Orientador
Departamento de Genética
Universidade Federal de Pernambuco – UFPE

_____

Prof. Dr. Luiz Bezerra de Carvalho Júnior
Departamento de Bioquímica
Universidade Federal de Pernambuco – UFPE

_____

Prof. Dr. Rafael Lima Guimarães
Departamento de Genética
Universidade Federal de Pernambuco – UFPE

_____

Profa. Dra. Maria de Mascena Diniz Maia
Departamento de Biologia
Universidade Federal Rural de Pernambuco – UFRPE

_____

Dra. Sandra Maria dos Santos
Polícia Científica de Pernambuco/SDS

Dedico esta pesquisa ao meu esposo Lucas Pacheco, aos meus filhos Luana Oliveira e Theo Oliveira, e a minha família.

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Obrigada a todos!

RESUMO

O uso de insetos visando responder aos quesitos levantados em investigações criminais ganhou espaço nas últimas décadas entre os pesquisadores e profissionais desta área, assim como a combinação de técnicas de genética forense para a obtenção de DNA humano a partir destes organismos, em especial dos dípteros necrófagos. Desse modo, neste estudo objetivou-se obter e testar um protocolo de identificação de DNA humano extraído a partir de larvas de dípteros coletadas em cadáveres no Instituto de Medicina Legal de Pernambuco Antonio Persivo Cunha (IMLAPC/PE). Inicialmente, espécimes imaturos foram coletados no IMLAPC/PE e criados em dieta a base de carne moída bovina para possibilitar a identificação da espécie mais abundante e frequente que se cria neste substrato. A espécie *Chrysomya albiceps* (Diptera: Calliphoridae) foi selecionada como modelo experimental. Grupos de larvas dessa espécie foram submetidos a uma dieta baseada em carne moída e sangue humano por 48 horas, dissecadas e submetidas a extração de DNA, utilizando-se duas metodologias comumente adotadas pelos laboratórios de genética forense: Kit DNA IQ™ e Método Fenol-Clorofórmio. O DNA extraído foi quantificado através de Nanodrop® e Real-Time PCR 7500 com uso do Quantifiler® Duo DNA Quantification. Para amplificação do DNA foram usados os kits para STR (short tandem repeats): AmpFℓSTR® Identifiler® Plus PCR Kit, Argus X-12® Kit e PowerPlex® Fusion System kit. As amostras amplificadas foram analisadas por eletroforese capilar em ABI PRISM 3500, permitindo observar que, para os kits utilizados houve perfis íntegros e compatíveis com a amostra referência, a partir da extração com kit DNA IQ™ e/ou método Fenol-Clorofórmio. Além disso, foram testados quatro meios de armazenagem comumente utilizados em zoologia: etanol 70%, etanol 95%, formol 4% e via seca. Após 24 horas de armazenagem, as amostras foram submetidas aos processos de análise de DNA e o formol 4% apresentou os melhores perfis de DNA. O fato de haver perfis passíveis de comparação confirma a utilidade das larvas de dípteros usadas para este fim, as quais podem futuramente ser usadas para correlacionar perfis genéticos com uma cena criminal. O aprimoramento destas técnicas é necessário para que o uso das larvas de dípteros muscóides com emprego para a entomogenética tenha mais difusão entre os meios acadêmico e forense.

Palavras-chaves: Genética Forense, Entomologia Forense, Diptera, Repetições de Microssatélites.

ABSTRACT

The use of insects for investigations has gained ground in recent decades among researchers and criminal professionals. Recently, the use of these animals has been combined with forensic genetics techniques for obtaining human DNA from these. Among the main focus groups for this technique are the carrion flies that have the host DNA extracted from intestinal contents. Because the visibility of this branch of forensic biology, this study aimed to obtain and test a protocol for identifying human DNA extracted from larvae of Diptera at the Instituto de Medicina Legal de Pernambuco Antonio Persivo Cunha (IMLAPC/PE). The species *Chrysomya albiceps* (Diptera: Calliphoridae) was selected as an experimental model. Groups of larvae of this species were subjected to diet ground meat and human blood for 48 hours, dissected and subjected to DNA extraction using two methods commonly used by forensic genetics laboratories: DNA IQ™ Kit and Method phenol-chloroform. The extracted DNA was quantified by Nanodrop® and Real-Time PCR 7500 with use of Quantifiler® Duo DNA Quantification. For DNA amplification kits for STR (short tandem repeats) were used: AmpFℓSTR® Identifiler® Plus PCR Kit, Argus X-12® Kit and PowerPlex® Fusion System kit. The amplified samples were analyzed by capillary electrophoresis in ABI PRISM 3500, allowing to observe for kits used there have upright profiles and compatible with the reference sample, from the IQ™ DNA extraction kit and/or phenol-chloroform method. In addition, four storage means commonly used in zoology were tested: 70% ethanol, 95% ethanol, 4% formaldehyde and dry. After 24 hours of storage, the samples were submitted to DNA analysis processes and the 4% formaldehyde DNA showed the best profile. The fact that there be comparable profiles confirms the usefulness of dipteran larvae used for this purpose, which can further be used to correlate genetic profiles and a criminal scene. The improvement of these techniques is required for the use of larvae dipterae with employment for the entomogenetics have more diffusion among academic and forensic means.

Key-words: Forensic Genetics, Entomology, Diptera, Microsatellite Repeats.

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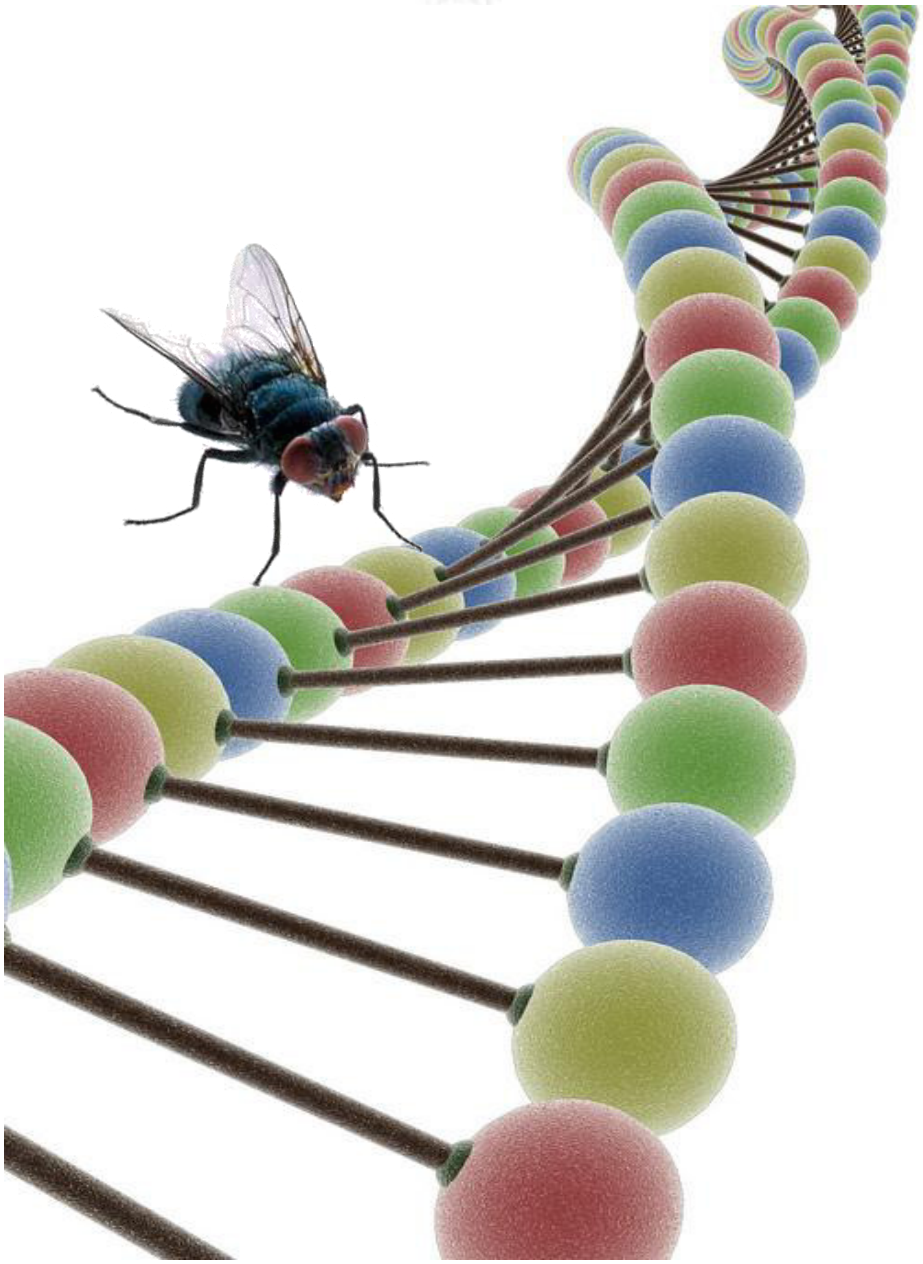
1. INTRODUÇÃO



1. INTRODUÇÃO:

Entomologia Forense é a ciência que aplica o conhecimento sobre insetos e outros artrópodes, seus processos e resíduos, como evidência em investigações criminais (Keh, 1985; Schroeder et al., 2003). Insetos são usados na ciência forense moderna desde o século XIX em casos de homicídios e investigações médico-legais (Benecke, 2001), sendo a sua principal aplicação a determinação do intervalo pós-morte (IPM) (Wells; LaMotte, 2001).

Dentre as diversas utilizações de insetos para responder questionamentos voltados as ciências forenses, uma recente aplicação vem chamando atenção: o uso do conteúdo alimentar de insetos necrófagos para recuperação de DNA humano (DiZinno et al., 2002). Esta união da entomologia com ferramentas da genética forense, pode ser chamada de entomogenética forense, um novo ramo no uso dos insetos para fins forenses.

No decurso das investigações forenses, muitas vezes há situações em que seria útil ter provas da ligação direta de uma larva com uma determinada vítima (Wells et al., 2001). Desta forma, insetos necrófagos e também hematófagos podem ser utilizados com sucesso para a recuperação de DNA humano, utilizando-se da reação em cadeia de polimerase (PCR) (DiZinno et al., 2002).

Os marcadores STR (*short tandem repeats*) de DNA nuclear, bem como as regiões hiperváriáveis de DNA mitocondrial (HVI e HVII) estão sendo comumente utilizadas para a análise do conteúdo alimentar de insetos (Li et al., 2011). Diversos trabalhos demonstram que, as amostras obtidas de insetos, tem qualidade e quantidade suficiente para gerar perfis de DNA humano capazes de servir como prova judicial (Kreike; Kampfer,1999; DiZinno et al., 2002; Wang et al, 2009; Li et al., 2011).

Dentre os insetos que participam ativamente da decomposição cadavérica, os dípteros muscóides são os mais utilizados para a recuperação de DNA humano, por serem os primeiros a chegar em cadáveres e carcaças, desde os momentos iniciais da morte (Carvalho et al., 2005). Os dípteros da divisão Cyclorrapha, que inclui Muscidae, Sarcophagidae e Calliphoridae,

possuem no seu trato digestivo um órgão de estocagem de alimento, o papo (Clery, 2001).

O papo é a área do trato digestivo das larvas de dípteros muscóides utilizada para extração e obtenção de perfil de DNA humano a partir do conteúdo alimentar, por não sofrer com o processo de digestão sobre o alimento ingerido (Hobson, 1931; Li et al., 2011). Campobasso et al. (2005) sugerem que as larvas do terceiro instar alimentando-se ativamente do cadáver podem ser consideradas uma ótima fonte de DNA humano.

O desenvolvimento de determinadas espécies em um cadáver, pode variar de acordo com as mudanças de temperatura e ambiente (Turchetto; Vanin, 2004). Informações sobre os insetos e seu crescimento em determinada região não devem ser seguidos à risca em locais com condições climáticas e geográficas diferentes (Amendt et al., 2004).

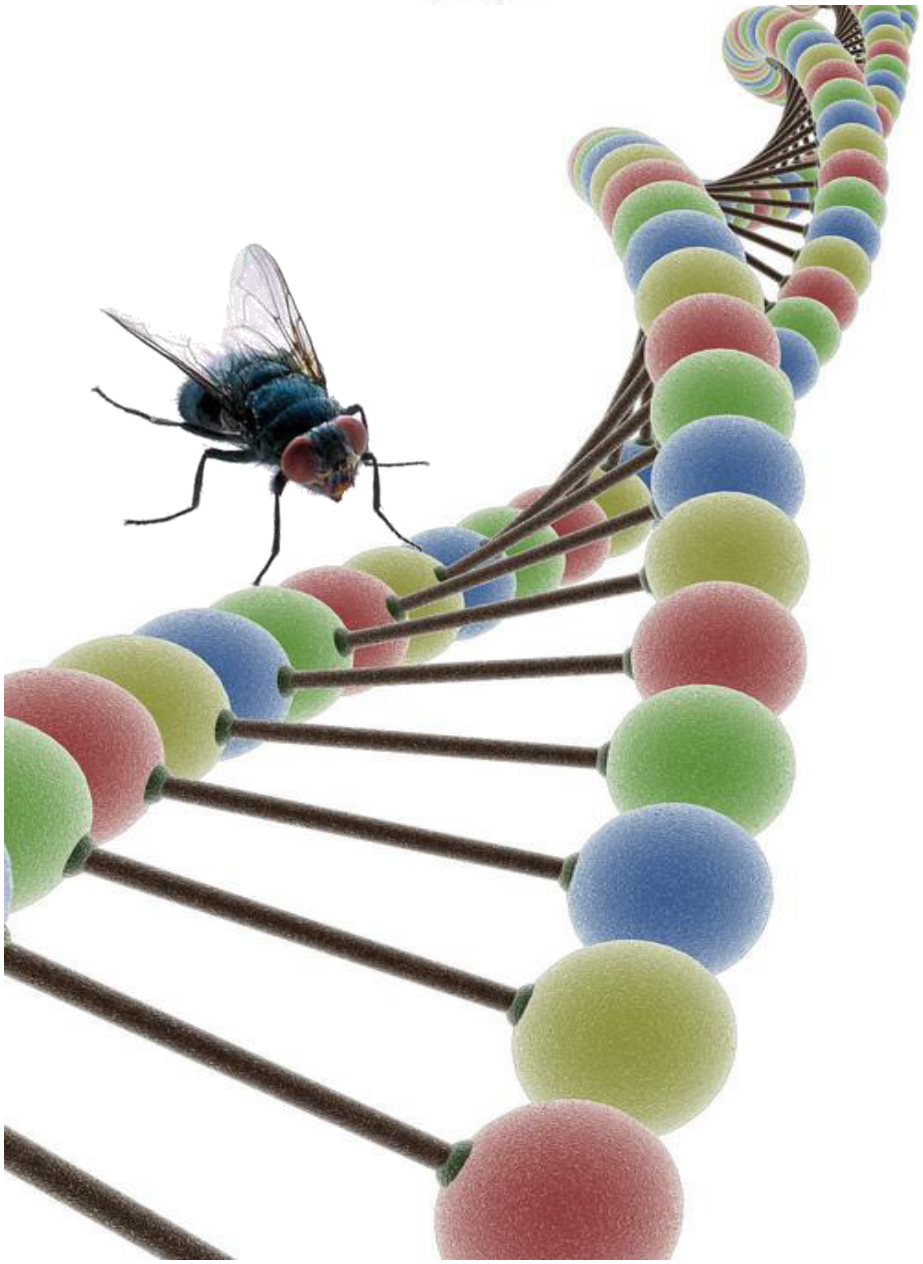
O mesmo deve ser considerado em relação a obtenção de DNA humano do trato alimentar de larvas, pois estas, em climas mais amenos ou mais severos, terão uma taxa de crescimento diferenciado, influenciando diretamente na velocidade do processo digestivo. Isso pode, não somente ocorrer entre locais, mas também entre espécies diferentes. Desta forma, os estudos que já tratam de técnicas para recuperação de perfil genético em insetos, precisam ser adaptados ao local onde será utilizado.

Também vemos na literatura uma escassez de padronização entre os estudos acerca do tema, fazendo com que o perito criminal, que não possui conhecimentos específicos da parte entomológica, venha a ter dificuldades em utilizar os insetos como uma ferramenta para a investigação.

Assim, objetivamos criar e padronizar um protocolo de obtenção de DNA humano retirado de larvas de dípteros coletados em cadáveres no Instituto Médico Legal de Pernambuco, que possa auxiliar na aplicação das técnicas utilizadas em entomogenética forense.

Para isso, visamos estabelecer uma colônia de dípteros de interesse forense para testes de obtenção de DNA humano *in vitro*; realizar testes de metodologias de extração de DNA de uso forense para padronização de protocolo; e analisar a efetividade de kits de âmbito forense para amplificação de DNA humano extraído a partir das larvas de Diptera.

2. APRESENTAÇÃO DO TRABALHO



2. APRESENTAÇÃO DO TRABALHO

A criminalística, conta com o auxílio de diversas ciências para reforçar uma investigação criminal. Hoje, a prova material é a principal ferramenta dos profissionais da área forense para buscar suspeitos e realizar associações entre pessoas, fatos e locais de crime.

Dentre essas ciências, aquelas ligadas à biologia forense, são umas das que mais ganham destaque, principalmente quando pensamos na prova advinda dos exames de DNA. Exames estes, que procuram identificar possíveis vítimas e agressores a determinado delito. Com a importância da prova do DNA, a área da genética forense começou a ganhar força em todo mundo, mobilizando profissionais acadêmicos e forenses na busca por técnicas e tecnologias que aumentem a confiabilidade e eficácia neste tipo de exame.

A cada dia, com o desenvolvimento de novas técnicas, produtos e usos de novos marcadores genéticos, podemos utilizar amostras de menor qualidade e com pouca quantidade de DNA para obter um perfil de DNA humano.

Ainda dentro da biologia forense, um outro ramo, chamado entomologia forense, que se utiliza de insetos como prova material, também está crescendo e ganhando espaço no mundo forense. Este ramo, aplica o conhecimento acerca da biologia e taxonomia de insetos para que estes possam atuar como vestígios em uma investigação.

Mais recentemente, técnicas que se apropriam do conhecimento da entomologia e das tecnologias da genética, vem ganhando destaque. É a entomogenética forense. Este ramo da biologia forense, utiliza o conteúdo alimentar de insetos necrófagos e hematófagos para recuperar DNA humano partir do trato digestivo desses animais.

Com o constante aumento da criminalidade no Brasil e, em especial na região Nordeste, a busca por ferramentas que auxiliem o trabalho da polícia, principalmente da perícia criminal e que visem encurtar o caminho da perícia e acelerar a produção do laudo pericial são necessárias, trazendo provas confiáveis e sempre baseadas em técnicas cientificamente validadas.

O Brasil possui poucas pesquisas utilizando a união da genética com a entomologia para fins forenses e, mais especificamente com insetos necrófagos. Com isso, visando aprimorar as informações acerca de insetos necrófagos, principalmente os representantes da Ordem Diptera, que são os mais importantes para as ciências forenses, para recuperação de perfil de DNA humano, essa tese propõe trazer conhecimento sobre a entomogenética forense que possam ser aplicados por peritos criminais e pesquisadores no estado de Pernambuco e somar aos que temos no mundo.

Desta forma, esta tese está dividida em formato de artigos, que facilitam a compreensão do trabalho realizado e ressaltam a diversidade de cada publicação. O primeiro artigo traz uma revisão de literatura sobre os hábitos alimentares, morfologia do trato digestivo e uma breve descrição do processo de digestão em dípteros muscóides.

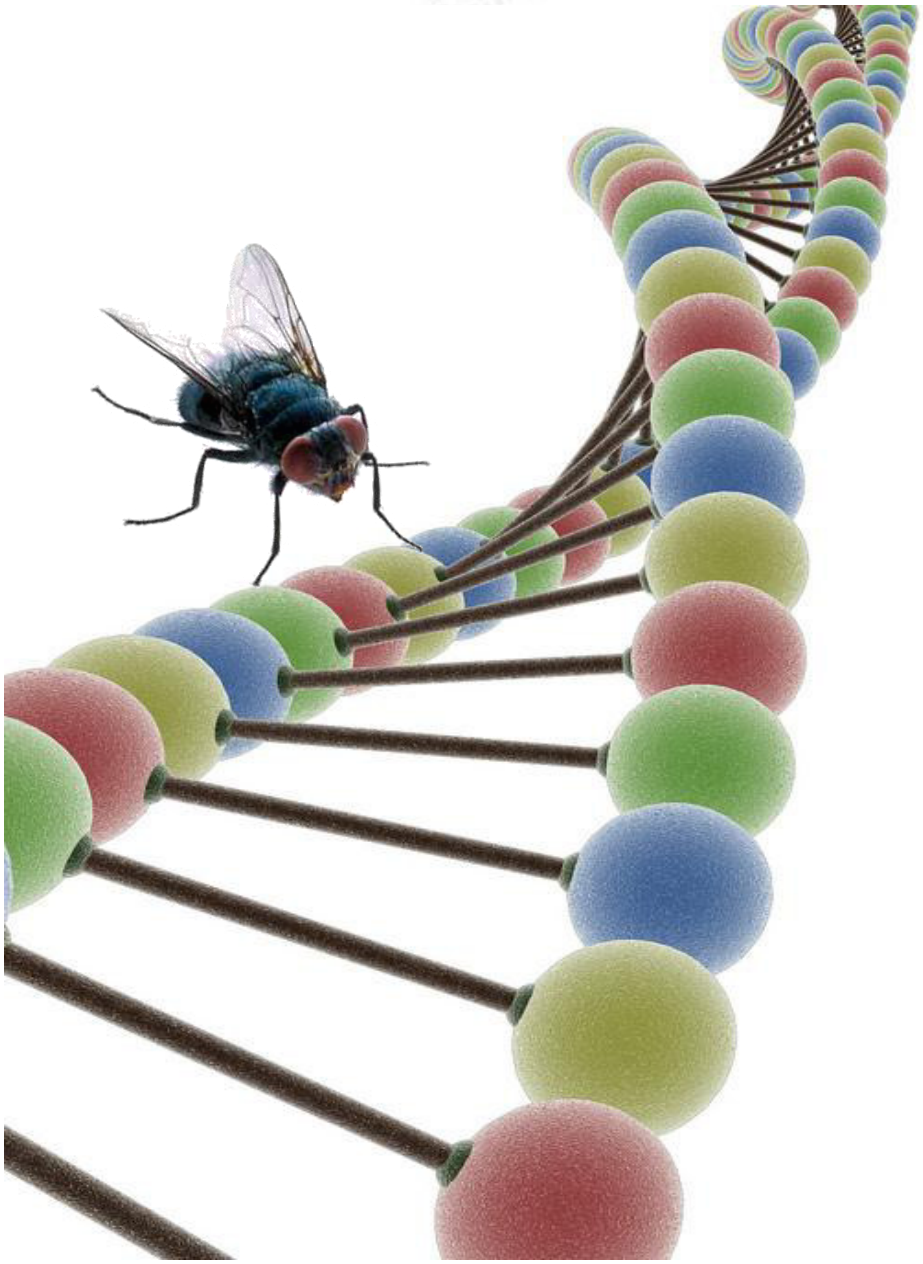
No segundo capítulo, temos a apresentação de casos reais envolvendo a colonização de cadáveres por insetos, acompanhados no Instituto de Medicina Legal Antônio Persivo Cunha em Pernambuco, com os quais foi possível iniciar os estudos entomológicos.

O terceiro, demonstra o processo de preparo de amostras de insetos para recuperação de perfil de DNA humano e as principais técnicas funcionais para este tipo de trabalho.

O quarto, traz conhecimento sobre as melhores metodologias de armazenagem do material entomológico para que haja a preservação do material genético no trato digestivo de modo que não prejudique a recuperação de DNA.

Para finalizar, em anexo, trazemos os principais protocolos testados e confirmados como eficazes para os exames genéticos em conteúdo alimentar de dípteros muscóides.

3. REVISÃO DE LITERATURA



3. REVISÃO DE LITERATURA:

3.1 – Ciências Forenses:

Dentro da Ciência Jurídica, a Criminalística destaca-se como a disciplina que tem por objetivo o reconhecimento e interpretação dos indícios materiais relativos ao crime ou à identidade do criminoso, trabalho este que até o século XIX era da Medicina Legal, que hoje trata apenas da investigação de fatores intrínsecos, ou seja, relativos à pessoa, cabendo a Criminalística o reconhecimento e interpretação dos indícios materiais extrínsecos contando para isso com o auxílio de diversas ciências (Tochetto et al., 1995).

A utilização de dados gerados por metodologias científicas é a base para a Criminalística, pois o material a ser analisado só poderá ser considerado válido se estiver baseado em dados científicos que possam ser testados e reproduzidos pelos mesmos exames e gerando os mesmos resultados, tudo isso baseado em diversas áreas da ciência (Velho et al., 2012).

Entre estas, destaca-se a Biologia Forense, que fundamenta diversos exames como: identificação de pessoas através de DNA; classificação de tipo sanguíneo e manchas de origem orgânica (sangue, esperma, fezes, urina, colostro); presença de substância psicotrópica ou tóxica e outros, além do uso da entomologia forense (Greenberg, 1991).

A incorporação da tecnologia de identificação de DNA forense para o processo de perícia criminal em um número crescente de países tem sido rápida e de longo alcance (Johnson et al., 2003). Conceitua-se que a Genética Forense emprega os conhecimentos da biologia molecular principalmente em relação a determinação de autoria de determinado crime e a materialidade dos mesmos (Francez; Silva, 2012).

Outro ramo da Biologia Forense, a Entomologia Forense de cunho médico-legal, é descrita como a utilização dos conhecimentos sobre insetos e outros artrópodes associados a um cadáver humano em decomposição com eventos envolvendo suspeita de crime, a fim de fornecer informações úteis para uma posterior investigação (Greenberg, 1991).

3.2 – Insetos Necrófagos e o Processo de Decomposição:

A putrefação é o processo de decomposição biológico da matéria orgânica e de acordo com a literatura médico-legal, desenvolve-se em quatro fases ou períodos distintos e consecutivos: o período cromático, o enfisematoso, o coliquativo e o de esqueletização (França, 2004).

Espécies de artrópodes que aparecem sucessivamente associadas a alterações em carcaças e cadáveres são principalmente necrófagas, sendo os dípteros os indivíduos mais predominantes (Keh, 1985). Insetos necrófagos são caracterizados por colonizar cadáveres, alimentando-se e depositando seus ovos ou larvas para que, na emergência de suas larvas, estas possam alimentar-se e completar seu ciclo de vida (Anderson, 2001). Sua atividade acelera a putrefação e a desintegração do corpo (Carvalho et al., 2004).

Os insetos, especialmente da Ordem Diptera, possuem órgãos sensitivos altamente especializados percebendo odores exalados pelos cadáveres, carcaças e restos mortais antes que possam ser percebidos por humanos, consequentemente sendo os primeiros seres vivos a chegar à cena de crimes e tendo a carne em decomposição como um excelente micro-habitat, servindo como sítio de cópula, estimulando a oviposição e atuando como principal fonte protéica (Catts; Goff, 1992).

Os dípteros são atraídos ao cadáver para ovipositar principalmente em lesões e em orifícios naturais como nariz, boca, ânus e vagina, onde as larvas passam em sequência, para o interior do corpo ajudando a degradar os tecidos moles, além de acelerar o processo de decomposição daquela região (Archer; Elgar, 2003). Sabe-se que o extravasamento de sangue, aberturas artificiais na pele e lesões causadas por agressões sexuais podem atrair mais os dípteros (Campobasso et al., 2001).

De acordo com Anderson (1997) e Grassberger e Reiter (2002), o clima influencia diretamente o desenvolvimento das espécies, acelerando-o em altas temperaturas e retardando-a em baixas. Isso faz com que o metabolismo e digestão dentre os espécimes varie, além de que, também os corpos em regiões tropicais possuem uma decomposição mais rápida do que nos climas temperados (Jíron; Cartín, 1981). Assim, dados sobre uma comunidade de insetos de uma determinada região não devem ser usados para outras

localidades de condições climáticas e geográficas diferentes (Amendt et al., 2000).

A taxa de desenvolvimento de insetos de modo geral, incluindo as larvas de dípteros muscóides, é influenciada pela temperatura do ambiente na qual se encontram e o desenvolvimento destes imaturos é de grande interesse para a ciência forense, especialmente para estimativa do Intervalo Pós-Morte (IPM) (Nuorteva, 1977).

O desenvolvimento de insetos pertencentes a Ordem Diptera ocorre por holometabolia, ou seja, possuem metamorfose completa, com o indivíduo passando por fase de ovo, larva, pupa e adulto (Triplehorn and Johnson, 2007) (Figura 1). A fase larval dos insetos é dividida em estágios, chamados de instares, que variam em número, de acordo com a espécie (Oliveira-Costa, 2011). Para os principais dípteros muscóides de importância forense a fase larval é composta por três instares (L1, L2 e L3), com duração entre estes estágios variando entre espécies (Gennard, 2007).

O instar larval e o tempo que o indivíduo leva para completar o ciclo de vida, são de extrema importância para a determinação do Intervalo Pós-Morte (Byrd; Castner, 2005). As fases de vida específicas das larvas podem ser identificadas pela número de fendas presentes nos espiráculos respiratórios localizados na porção posterior (Gennard, 2007).

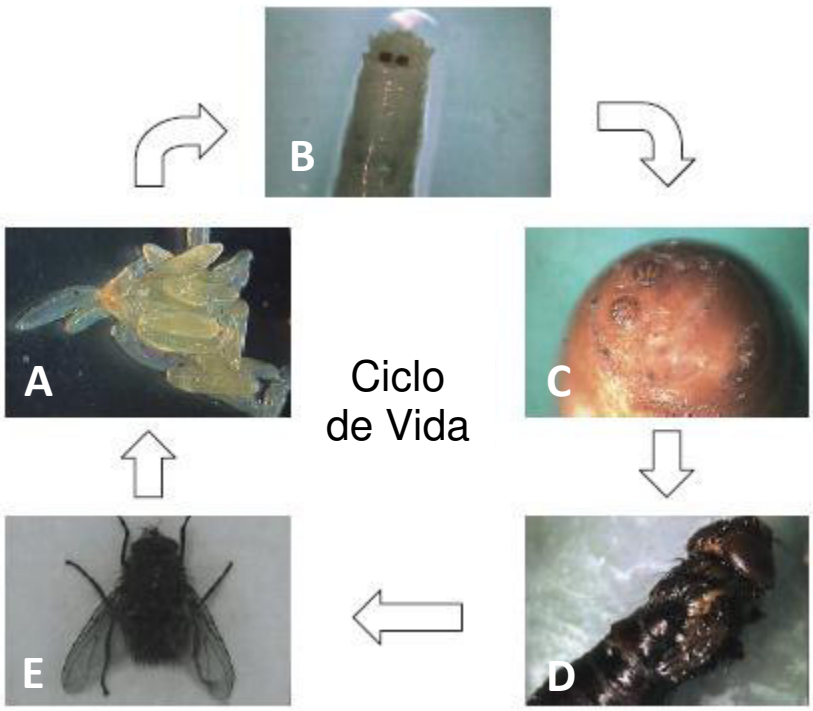


Figura 1: Ciclo de vida de um díptero muscóide com as fases de desenvolvimento: A- ovos, B- larva, C- porção final demostrando os espiráculos respiratórios que auxiliam na estimativa da idade larval; D- adulto recém emergido deixando o pupário; E- díptero adulto. Fonte: Gennard, 2005.

3.3 – Morfologia de Imaturos de Dípteros Muscóides e de seu Trato Alimentar:

A grande maioria das larvas de dípteros muscóides é saprófaga, ou seja, alimenta-se de matéria orgânica em decomposição, e a quantidade de alimento, bem como sua necessidade nutritiva pode ser alterada nos diferentes instares, além da sua estrutura que pode variar significativamente de acordo com as condições de alimentação (Oliveira-Costa, 2011).

As larvas geralmente são cilíndricas com cabeça pontiaguda e compostas por 12 segmentos: cabeça (segmento 1), protórax (2), mesotórax (3), metatórax (4) e oito segmentos abdominais; espiráculos respiratórios posteriores formados por fendas, que variam de acordo com o instar larval, estão localizados no último segmento abdominal; estendendo-se da cabeça ao tórax, temos um esqueleto cefalofaringeano quitinoso, que consiste em diversos escleritos distintos; espiráculos anteriores (quando presentes) estão em cada lado do protórax (Thyssen, 2010) (Figura 2).

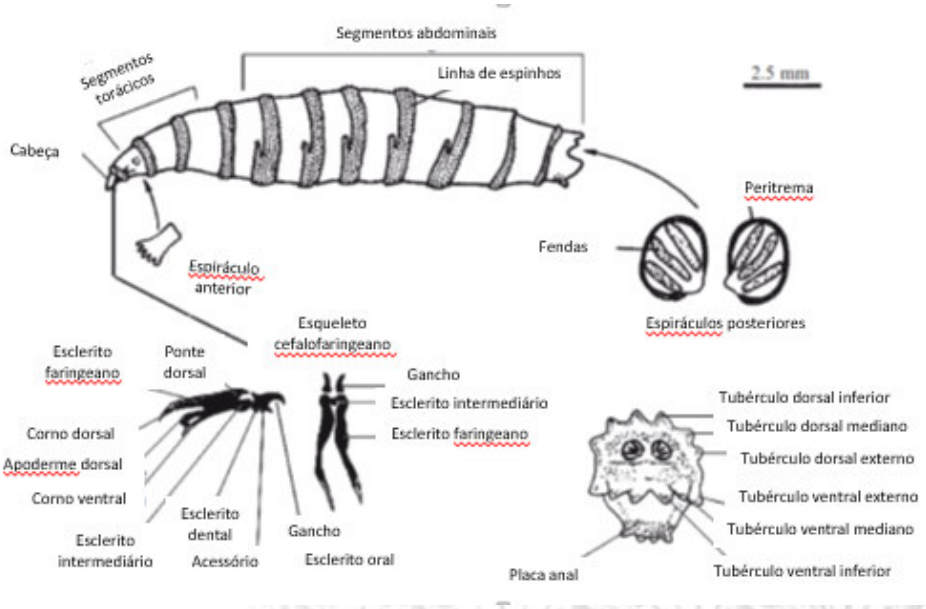


Figura 2: Morfologia de larva de díptero muscóide mostrando seu esqueleto cefalofaríngeano e espíraculos respiratórios. Fonte: Thyssen, 2010.

O canal alimentar das larvas de dípteros muscóides possui três regiões principais: o intestino anterior ou estomodeu, o intestino médio ou mesêntero e o intestino posterior ou proctodeu (Triplehorn; Johnson, 2007).

O intestino anterior é composto pela boca (Mo), glândulas salivares (SG), esôfago (Es) e papo (Cr); a cárdia (Ca) representa a junção do intestino anterior e médio que consiste em ceco gástrico (GC), seguido pelo intestino anterior médio (AMG), meio do intestino médio (MMG) e intestino posterior médio (PMG); o intestino grosso (HG) começa com o piloro (Pi), a partir do qual os tubos de Malpighi (MT) surgem, íleo (Il), cólon (Co), reto (Re) e ânus (An) (Boonsriwong et al., 2007) (Figura 3).

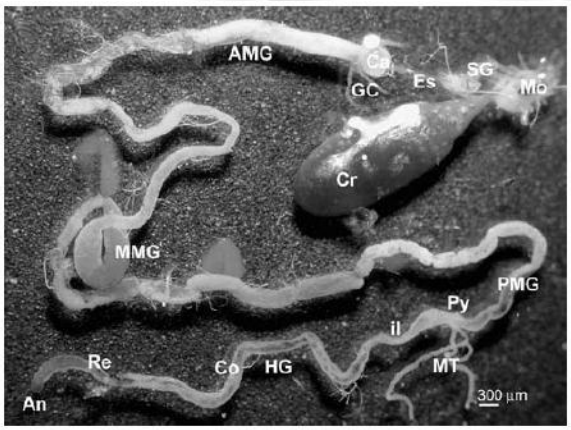


Figura 3: Canal alimentar de larva de *Chrysomya megacephala* (Diptera: Calliphoridae) em terceiro instar. Fonte: Boonsriwong et al., 2007.

O papo, localizado no intestino anterior, é uma estrutura para armazenamento do alimento, que poderá sofrer alguma digestão em insetos no qual a saliva contém enzimas (Gillott, 2005). Hobson (1931) já mencionava que quando as larvas eram alimentadas com sangue fresco, ocorria um escurecimento no centro do segmento anterior ao intestino médio e salienta que o papo aparenta funcionar para armazenagem, pois a larva faminta passa o primeiro alimento diretamente para o intestino médio, sem deixá-lo no papo (Figura 4).

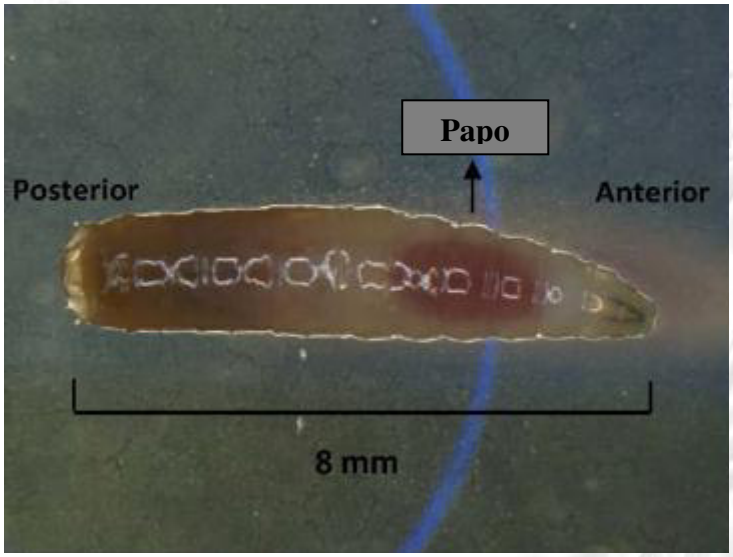


Figura 4: Larva da família Calliphoridae com alimento estocado no papo, sendo visível a olho nu. Fonte: Kondakci et al., 2009.

3.4 – Entomologia e DNA:

O uso mais difundido da Entomologia Forense é para estimar a cronologia da morte, o chamado intervalo pós-morte (IPM), que visa estabelecer o tempo mínimo e máximo, entre a morte e o momento em que o corpo foi encontrado (Bornemissza, 1957; Schroeder et al., 2003).

Porém, há inúmeras situações em que os insetos podem ser usados como evidência ou prova física em investigações, estando a pessoa viva ou morta como, por exemplo, elucidar questões relativas à movimentação do corpo, identificação por DNA, lesão no cadáver ou manchas de sangue (artefatos), local de morte, investigação toxicológica, negligência no cuidado a

crianças ou incapazes, identificação de suspeitos que tenham ligação com a cena do crime através de DNA, entre outras (Goff; Lord, 2001).

Sabe-se que um genótipo humano pode ser recuperado a partir de um mosquito alimentado ou de um piolho transferido durante um crime sexual, apesar de alguns deles terem digestão oral parcial, o conteúdo intestinal de uma larva de díptero muscóide pode ser apropriado para todos os típicos procedimentos de identificação genética (Wells; Stevens, 2008).

Em certas circunstâncias, a análise de DNA de insetos, pode ser importante para comprovar a associação de larvas específicas de um cadáver, como casos em que, nenhum corpo foi descoberto, porém existem larvas, pode-se desencadear uma investigação criminal se a análise desses imaturos apontar a presença de DNA humano proveniente daquele local (Zehner et al., 2004). Pode-se ainda utilizar as larvas em casos de crime sexual, onde os imaturos poderão se alimentar não somente de material da vítima como também do sêmen do agressor (Clery, 2001).

De Lourdes Chavéz-Briones et al. (2013) relataram recentemente um caso de identificação humana baseado em larvas obtidas de um cadáver que se pode comparar com material genético doado pelo pai da suposta vitima. A análise genética de conteúdo intestinal de insetos pode provar ser evidência crucial para ligar um suspeito à vítima ou a cena, reconstruir as circunstâncias do crime, ou estabelecer a credibilidade das declarações feitas por testemunhas (Campobasso et al., 2005).

3.5 – Marcadores STR e Identificação Humana:

Os Marcadores STR (*Short Tandem Repeats*) consistem de sequências repetidas, entre 2 e 6 pares de bases (pb), que apresentam um grau elevado de polimorfismo devido à variação do número de unidades de repetição, sendo as de pelo menos 4 pb usadas para análises forenses (Lygo et al., 1994, Ruitberg et al., 2001). Foram utilizados pela primeira vez no tratamento de casos forenses no início da década de 1990 e até o final desta haviam se tornado uma ferramenta padrão para quase todos os laboratórios forenses do mundo (Goodwin et al., 2007).

As sequências repetitivas de DNA, STRs ou microssatélites, são amplamente utilizados na identificação humana, sobretudo em casos criminais e desastres em massa (Ye et al., 2004). A capacidade de extrair DNA em quantidade, qualidade e integridade satisfatória é uma exigência indispensável nas análises de DNA para identificação de indivíduos (Álvarez et al., 2001).

A análise genética de loci STR é atualmente o método mais eficaz e largamente utilizado para identificar amostras biológicas recolhidas no âmbito das investigações criminais, bem como a identificação de restos humanos, sendo a aplicação bem sucedida para uma variedade de substratos tais como sangue, saliva, células epiteliais, raízes de cabelo e amostras de osso (Schneider et al., 2004).

Os microssatélites vêm sendo comercializados e largamente utilizados nos laboratórios sob a forma de kits que representam um conjunto de marcadores moleculares usados para identificação humana, previamente validada, os quais são amplificados juntos em uma mesma reação, numa mesma PCR (Collins et al., 2004). Pesquisas demonstram que é possível obter dados de STR atráves da alimentação de sangue de um inseto adulto, do mesmo modo, estudos em laboratório confirmam que o conteúdo intestinal de larvas pode ser usado para obtenção de perfil STR humano (Zehner et al., 2004).

Para explorar o potencial dos loci STR, o FBI patrocinou um esforço de toda a comunidade científica para selecionar e estabelecer o CODIS (isto é, Combined DNA Index System), resultando em 13 loci: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX e vWA (Budowle et al., 2001) (Figura 5).

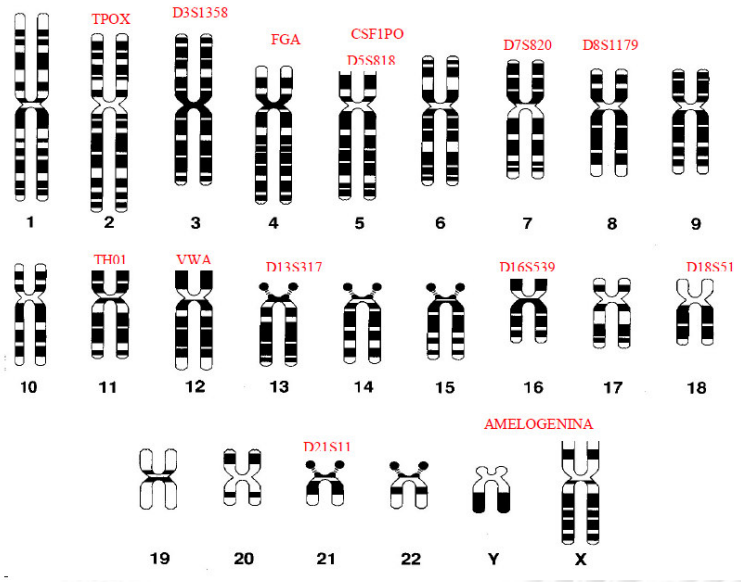


Figura 5: Posição cromossômica dos 13 loci CODIS recomendados para identificação humana com fins forenses. Fonte: Santos, 2010.

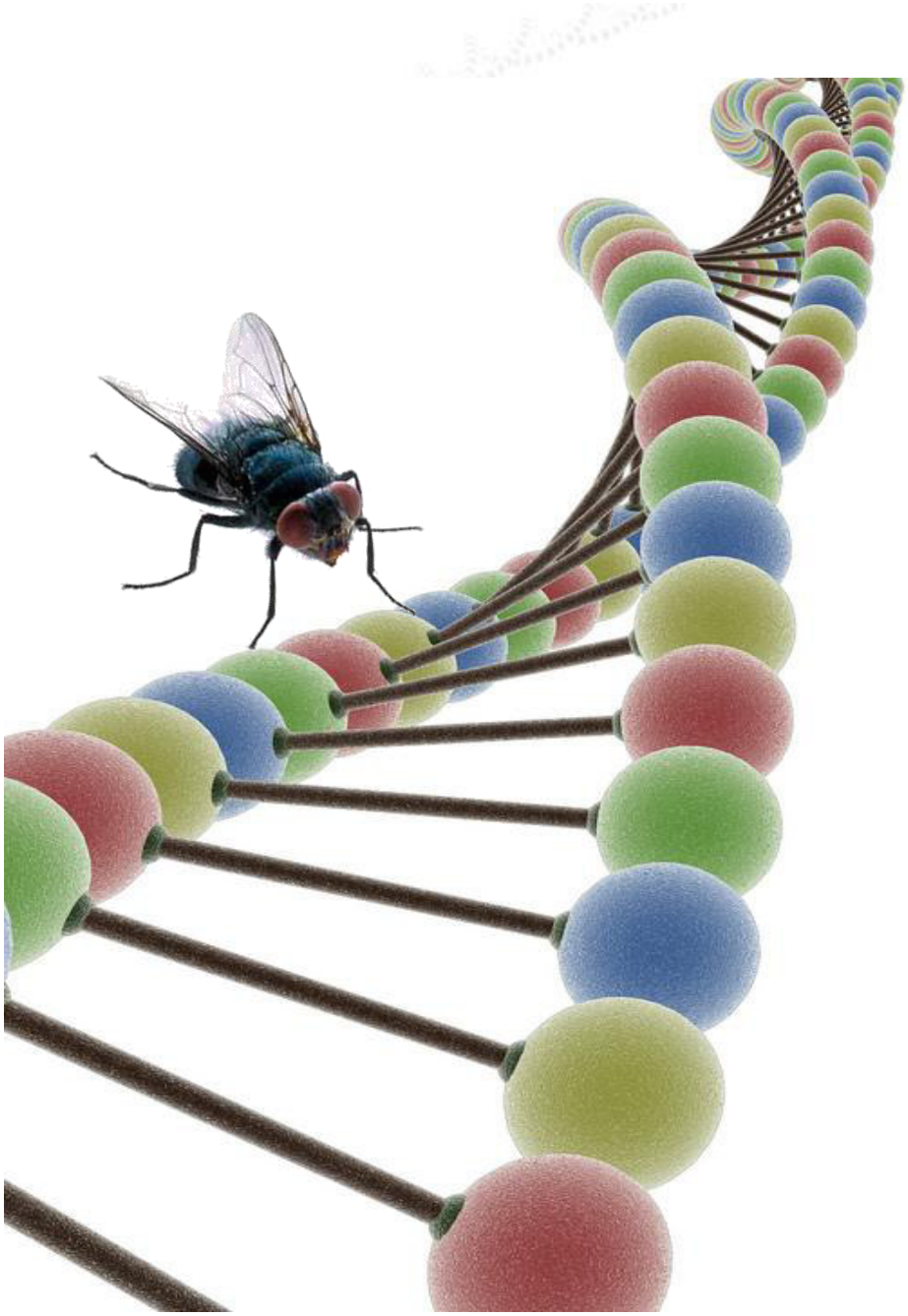
Em 2010 o FBI realizou uma revisão e expansão do número de loci para o sistema CODIS com o intuito de: reduzir as probabilidades de correspondências, pois o número de perfis armazenados aumenta a cada dia; aumentar a compatibilidade internacional para a partilha de dados; e aumentar o poder de discriminação em casos de pessoas desaparecidas (Hares, 2012). De acordo com a publicação, os seguinte loci foram incorporados ao sistema CODIS: D2S1338, D19S433, D1S1656, D12S391, D2S441, D10S1248, Penta E, DYS391, D22S1045, SE33, Penta D, totalizando 24 loci STR.

Quando amostras mais problemáticas tais como manchas antigas ou tecidos em decomposição são submetidas a tipagem STR, pode-se não obter resultados reprodutíveis devido a degradação do DNA (Schneider et al., 2004). Os cientistas forenses muitas vezes enfrentam o problema da extração e tipagem do DNA humano a partir de materiais degradados, tais como músculo e os ossos de corpos decompostos (Piccinini et al., 2006). Ossos e dentes, dependendo das circunstâncias, podem conter pouco DNA (Wilson et al., 1995). Amostras ósseas são particularmente difíceis e demoradas para serem analisadas e outros tecidos corporais sofrem rápida deterioração (Piccinini et al., 2006).

No caso de investigações de crimes, a degradação do DNA humano é normalmente o resultado de um processo natural resultante da exposição das

amostras de manchas ou do tecido humano ao ambiente (Schneider et al., 2004). Amostras de DNA humano obtidas a partir do conteúdo intestinal de insetos, serão num futuro próximo, uma alternativa para as invetigações criminais (Wells; Stevens, 2008).

4. REFERÊNCIAS BIBLIOGRÁFICAS



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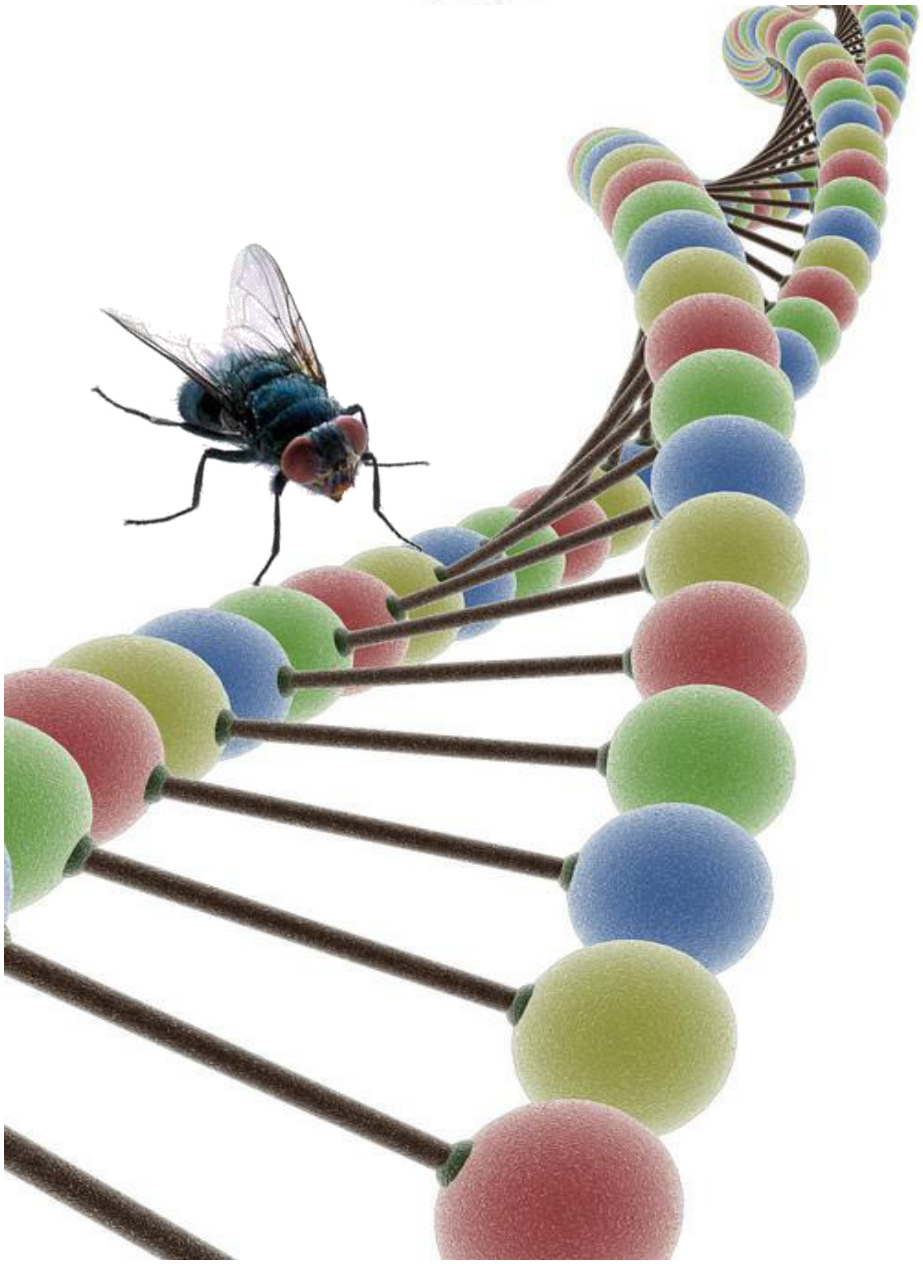
5. PRODUÇÃO CIENTÍFICA



5. PRODUÇÃO CIENTÍFICA:

5.1 – Artigo 1

Digestion in necrophagous dipterians larvae and their implications for forensic entomogenetics - a review

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Periódico: Journal of Insect Physiology (Submetido)

Digestion in necrophagous dipterians larvae and their implications for forensic entomogenetics - a review

Tatiana Costa de Oliveira[1*], Sergio Crovella[1]

[1]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, Cidade Universitária, Recife, Pernambuco, Brazil.

*__Corresponding author__
Tatiana Costa de Oliveira
E-mail: ttattian@yahoo.com.br.
Address: Rua André Dias Figueiredo, 120, ap. 102, Barro, Recife, Pernambuco, Brazil, CO 50780-080.
Tel: +55-81-3251.0584 / +55-81-98726.2637. Fax: +55-81-2126.8485

ABSTRACT

The use of insects in criminal investigations is already widespread among criminologists and researchers, however, a new applicability in the use of these animals, forensic entomogenetics, has been gaining attention. This most recent field of forensic entomology concerns the use of human DNA recovered from the insect digestive tract. The use of insect scavengers for this purpose is a real possibility but requires prior knowledge about the biology and habits of these specimens. Consequently, it is necessary knowledge of the anatomy and physiology of these necrophagous insects so getting this DNA profile occurs in quality and quantity to generate a satisfactory criminal evidence. This work aims to contribute to the knowledge of the anatomy and physiology of dipterae scavengers through a review of morphology, feeding behavior and digestive process in this group of individuals.

Keywords: Forensic Entomology, Digestive System, Diptera, Enzymes, DNA.

1. INTRODUCTION

Forensic entomology uses the knowledge about the habits and insect behavior to generate a criminal trial (Keh, 1985). Among the insects that are commonly used for forensic purposes, we can highlight those of scavengers habits, especially representatives of the order Diptera (Anderson, 2001; Campobasso et al., 2001).

The representatives of this Order, stand up before other groups by being the first to reach the body or housing, and actively participate in the decomposition process (Catts and Goff, 1992). By being the first to colonize a body, it is the main tool for estimating the postmortem interval, the main application of forensic entomology (Schroeder et al., 2003).

The applications of insects with forensic purpose go far beyond the estimated time of death. Other applications are highlighted, such as the determination of the place of death or drug detection, but another use, the human DNA recovery from the digestive tract of flies scavengers is growing among practitioners and researchers in the forensic field (Goff and Lord, 2001; Zehner et al, 2004).

This obtain a DNA profile from a scavenger flies' larvae require a prior knowledge of the morphology and physiology of the digestive tract such specimens. Specific regions of the digestive tract must be used to obtain the DNA sample, and therefore requires a certain level of professional knowledge about the methodologies to be used for this purpose.

Further studies on the digestive system carrion flies are few, especially when it comes to diet, digestion and excretion of these insects in the larval stage. The objective of this review is to demonstrate the digestive behavior of muscoid Diptera larvae and its implications for use in forensic entomogenetics.

2. REVIEW

2.1. INSECTS SCAVENGERS

Insects always remarkable for its diversity of habits and habitats, are recognized as important vectors of pathogens, urban and rural pests, pollinators etc (Zumpt, 1965; Triplehorn and Johnson, 2007). Due to the different habits, a group of insects is highlighted by effectively acting on cadaverous decomposition process and are used in forensic entomology (Bornemissza, 1957).

The forensic entomology is the application of insects to assist in the legal field investigations (Schroeder, 2003) and can be classified into three categories: urban, when there is the presence of insects, like termites in buildings, damaging them; the stocked products, which relates to contamination of stored products such as beetles beans; the forensic involving the criminal area, especially in relation to violent death and uses necrophagous insect as an aid to investigations (and Lord Stevenson, 1986).

The main application of forensic entomology is the estimated timing of death, called the postmortem interval (PMI), which aims to establish the minimum and maximum time between death and the time the body was found (Bornemissza 1957; Schroeder et al, 2003).

This calls for a study of the succession of these insects on cadavers and carcasses during the decomposition, with preference for certain condition of the corpse (Campobasso et al., 2001). Each cadaverous decomposition stage is characterized by certain insect groups, each occupying a specific niche, and its activity and abundance influence the physical properties of the body, the speed of decomposition, seasonality and abiotic conditions (Aggarwal, 2005).

These groups, known as sarcosaprophagous, ie, animals participating in the transformative process of corpses and carcasses are classified according to their habit and can usually be divided into four ecological groups (Nuorteva, 1977). Most species listed as participating in the decay process, are a further obstacle to the reduction of waste, since many are on the body to prey insects found scavengers (Fichter, 1949).

According to Campobasso et al. (2001), the groups are divided into: necrophagous, that colonize the bodies, feeding directly from dead tissue; predators and parasitoids, feed on insects are colonizing the body or use the reserves of the body of settlers to complete its life cycle; omnivores feed on both decaying matter, as the fauna associated with this; acidental, they are on the corpse by chance and use it as an extension of their habitat, visiting him occasionally.

The insects, in many situations, are the first to arrive at crime scenes, this because possess specialized sense organs and can detect odors exhaled by corpses, carcasses and remains much before they can be perceived by humans (Catts and Goff, 1992).

Among the insects that part of cadaverous decomposition, we can highlight the representatives of the Order Diptera and Coleoptera as the most important for the transformative process of the corpse (Souza and Linhares, 1997). One of the most important aspects of the corpse decomposition is the role of larvae of dipteran scavengers, especially blowfly, inside the housing (Putman, 1977).

In the initial stages of decomposition, the flies tend to predominate, making it the most important group for forensic use, but when the body is already in the final stages of skeletonization, a second group of great importance, Coleoptera, becomes more a tool for calculating the postmortem interval (Kulshrestha and Satpathy, 2001).

2.2. DIPTERA FORENSIC IMPORTANCE

The infraorder muscomorpha is defined to include only the Brachycera cyclorraphous where the main species of forensic importance are inserted, as members of the superfamily Oestridae (MacAlpine, 1987). The Oestridae superfamily includes five families, among which are the families Calliphoridae, Sarcophagidae and Muscidae, which has great forensic importance (Gillott, 2005).

Since the report of Mégnin (1894), the synanthropic flies, especially representatives of the Calliphoridae family, has been considered as the first wave in faunal succession of cadaverous decomposition (Grassberger et al., 2003).

The flies belonging to the family Calliphoridae, known popularly as blowflies, and the Sarcophagidae family, are considered the most important consumers of carcasses and corpses, living in semi-liquid medium and generally staying on decaying matter until complete their life cycle (Reed, 1958; Braack, 1987; Lord and Goff, 1990; Catts and Goff, 1992).

Specimens are usually involved in estimating the postmortem interval are flies of the Calliphoridae, Sarcophagidae and Muscidae families also used as the main tools for applicability of forensic entomology, being agile, good flying and typically the first to arrive on carcasses and corpses (Goff and Lord, 1994).

Among these families, Calliphoridae is considered the most important for studies involving Forensic Entomology, being the most widely studied group of flies for these purposes. This family is within the tribe Chrysomyini and represented in the New World for 20 endemic species and four species introduced: the endemic are in five genera (Chloroprocta, Paralucilia,

Hemilucilia, Cochliomyia and Compsomyops) and introduced through the old world, are encompassed in the genera Chrysomya (Dear, 1985).

According to Carvalho and Ribeiro (2000), the list described by James (1970) lists at least 100 species of blowflies to the Neotropics and Pont (1980) reports that there are 1 020 species in all biogeographical regions.

The family Calliphoridae larvae may have biontophagous or necrophagous habits, may cause myiasis mandatory or voluntary, as well as acting in cadaverous decomposition process (Carvalho and Ribeiro, 2000). Species of arthropods which appear successively in correspondence with the phases of corpse decomposition are mainly scavengers, blowflies being the most significant (Keh, 1985).

Carcasses and rotting corpses are an ephemeral and unequal resource and insects that feed there, rarely complete more than a generation in the body (Wells and Greenberg, 1992). Calliphoridae flies females can find and lay eggs on a corpse within minutes after death, except when there are barriers or unfavorable environmental conditions (Slone and Gruner, 2007).

After death, the body has a variety of tissues and organs available to serve as oviposition substrate for colonization and scavenger flies (Kaneshrajah and Turner, 2004). The vast majority of females flies not oviposit in dehydrated or mummified tissues, as eggs and larvae need liquid or soft tissues to develop (Introna and Campobasso, 2000).

Insects seek lay their eggs or larvae in natural orifices as ears, mouth, nose and eyes or open wounds, accelerating the decomposition process of the region (Archer and Elgar, 2003). Particularly, soft tissues such as lung and brain, are often attacked first and consumed more rapidly than others (Kaneshrajah and Turner, 2004).

2.3. THE DIGESTION IN LARVAE OF DIPTERA NECROPHAGOUS

The larvae of Cyclorrhapha more recently evolved into "worms" with an entirely invaginated head and chest, all parties, except for parts of the jaw and cephalopharyngeal skeleton, are esclerotizated (Grimaldi and Engel, 2005). In fact, the larvae of muscomorpha, seem to have only a new feeding mechanism,

but a changing roles of mouthparts that are suppressed in young stage (Gillott, 2005).

The shape of the gut and the complexity of its structure are related to the eating habits of insects. Those who feed on blood, sap, nectar and exudates, have a long digestive tract, narrow and folds to facilitate contact with the liquid food (Gallo et al., 2002).

Most foods that require digestion by insects consisting of polymers including starch and cellulose, forming glucose units linked together by alpha and beta; hemicellulose, which is a mixture of polymers linked by beta bonds polymers of monosaccharides; and proteins, which are chains of amino acids (Terra, 1990). The necrophagous insects used mainly from the digestion of proteins which are obtained in decomposing organic animal matter.

Cyclorrhapha larvae has an innovative lifestyle compared to other flies: they live in their food and are not limited to food in the aquatic environment, but explore a smorgasbord of meats and fruits. These use the jaw pieces to remove the substrate while secrete saliva abundantly a soup by ingesting oral cavity (Grimaldi and Engel, 2005). Larvae can only take liquid foods, because their mouth parts are not adapted for mastication (Hobson, 1931).

The alimentary canal is divided into three parts: the stomodeum or foregut, the proctodeum or hindgut and midgut (Triplehorn and Johnson, 2007). In the foregut, right after the oral cavity, we have the region of the pharynx, where is located the cephalopharyngeal skeleton, and soon after the crop emerges, where the food is stored for some time (Gallo et al., 2002).

The crop and midgut have the highest concentration of bacteria. A gradual decrease in the number of bacteria could be seen throughout the intestine, although some sections with few bacteria are followed by areas with large numbers of micro-organisms (Mumcuoglu et al., 2001).

The midgut has a peritrophic membrane composed of proteins and divided into endoperitrophic space (within the matrix) and ectoperitrophic (adjacent to the epithelium of the midgut) (Gillott, 2005). There is a movement of digestive enzymes endo-ectoperitrophic, which can be driven by fluid flow, observed in the midgut and was suggested by the finding that an increase in

dietary protein fed larvae led to a decrease in trypsin gradient along midgut with trypsin increased excretion rate (Terra, 1987).

Water and amino acids are absorbed principally by initial portions and midgut average, respectively. This water is released at the posterior midgut and feeds endo-ectoperitrófica movement, making the movement of digestive enzymes and transporting products of digestion (Terra, 1990).

Many insects, such as Sarcophagidae flies, release digestive enzymes on food and may occur partial digestion before the food is brought to the body (Triplehorn and Johnson, 2007). It has been proven healing properties in dipteran larvae and such properties are due to the activities of enzymes contained in waste products of excretions and secretions (ES) released by larvae (Pinilla et al., 2013).

These compounds excretion/secretion released by the salivary glands and intestine into the surrounding tissue exhibit a wide antimicrobial activity (Valachová et al., 2014). One of the best antibacterial compounds described and found in ES products is lucifensin a larval defensin expressed in the salivary glands of all larval stages (Valachová et al., 2013).

The serine proteases are characterized by having a nucleophilic serine residue in its active site (that is, the catalytic triad) containing histidine, asparagine and serine amino acids (Hedstrom, 2002). These proteases have broad substrate specificity, which is consistent with the biological role played in most insects, such as the establishment and supply of nutrients for the larvae (Young et al., 1996; Angulo-Valadez et al, 2007, Pinilla. et al., 2013).

The chymotrypsin and trypsin are the most common enzymes in the family of serine proteases that have been characterized and can be found primarily in the insect digestive systems (Mazumdar-Leighton and Broadway, 2001).

It has been reported that the major proteolytic activity in the second and third larval stage may be associated with greater weight gain after the change (Cepeda-Palacios et al., 1999), where the larvae has gained about 45% of its weight average adult (Pinilla et al., 2013).

It is possible that during the course of the alimentary tract, the intestinal content is moved. The number of bacteria decreases significantly in the anterior

part of the large intestine, and practically no bacteria is observed on the rear end near the anus (Mumcuoglu et al., 2001).

The cropt appears to function as a storage organ once the larvae have food starving directed directly to the midgut (Hobson, 1931). Roberts (1971), reports that most of the particles are bacteria found in the crop, and it is believed that these are the major food source of the larva.

Dead bacteria and fungi can be considered as the primary feed and vitamin resource fly larvae that feed on decaying matter, such as blue bottle fly species considered, in fact, mycetophagous (Roberts, 1971). The midgut acidic zone may be responsible for killing micro-organisms acquired in releasing nutrients to feed the larvae with the aid of a cathepsin, D-like enzyme and an acid lysozyme (Terra and Ferreira, 1994).

Among the insects, the ability to digest bacteria in the midgut, appears to be an ancestral feature of cyclorrhaphous dipteran, which supports the latest reputation of saprophagic larvae feeding in large part, of bacteria (Lemos et al., 1993).

According to studies, Cyclorrhapha larvae living in highly infected food, drink large quantities of bacteria, which constitute their main food and once ingested, are killed their in midgut by the combined action of low pH, lysozyme and acid proteases (Terra and Ferreira, 1994). Thus, nutrients are passed to the hindgut to the rest of digestion occurs.

The bacteria in foods that were killed by the combined action of lysozyme and low pH in the midgut, release several proteins that are first digested by trypsin in the midgut lumen (Terra, 1990).

Hobson (1931) reports that when the larvae are fed with fresh blood, one can observe a darkening that occurs in the middle of the midgut segment being in chat the still red blood. The darkening of the blood in the middle segment is probably due to hydrolysis of hemoglobin to hematin and globin and not digest itself.

The absence of digestion in the anterior segment of the digestive system, despite the presence of enzymes is a favorable reaction (Hobson, 1931), especially when the larvae are used for forensic purposes.

2.4. FORENSIC ENTOMOGENETICS AND USE OF LARVAL INTESTINAL CONTENTS

The DNA typing has been used for forensic purposes in different types of samples and facilitated by PCR techniques (Wilson et al., 1995). The application of techniques to obtain DNA focused on forensic entomology emerged with the need to seek more information about the taxonomy of species used as tools in criminal investigations (Benecke, 1998; Wallman and Donnelland, 2001).

New applications for DNA typing in entomology arose from the use of hematophagous insects to obtain a human profile from these individuals (Lord et al., 1998). In forensic cases of bodily harm and sexual assault, the analysis of biological stains is of great importance to clarify the circumstances of the crime and identifying the offender (Kreike and Kampfer, 1999).

Forensically, such analyzes can help identify perpetrators in cases of violent crimes (such as rape, murder and child abuse), where the insects that feed on blood or their droppings are recovered from victims or crime scenes can be a great tool for research (Lord et al., 1998). When we talk about carrion insects, we think of its use to estimate the time of death and in such cases it is necessary that the association of the larvae of flies to a particular body is made more effectiveness (Wells et al., 2001).

For this, more research is bringing techniques for obtaining human DNA from samples from the gastrointestinal tract associated with insects scavengers decomposing tissues (DiZinno et al., 2002).

We can use the food contents collected from scavengers flies larvae for some situations like when we larvae, but we found no close corpse; or, when attached to the larvae have more than one feed source (two bodies, for example); or when there is no certainty of the link between the larvae and the body (Wells et al., 2001; Linville et al., 2004).

In these circumstances, the DNA analysis of human material ingested by the larvae could produce a genetic profile suitable for comparison with the corpse (Zehner et al., 2004). The isolation method used to obtain the DNA of the host from larvae is important because it should not result in further

degradation of DNA, and should provide a sufficient quantity of detectable DNA and used in subsequent molecular procedures (Carvalho et al., 2005).

During the digestion process, the liquefied tissue of the host are temporarily stored in a special area before the larval gut, the crop (Zehner et al., 2004). Despite its preoral partial digestion, bowel contents dipterous larvae can be suitable for all genetic procedures typical of human identification (Wells and Stevens, 2008).

Within the blowfly larvae, for example, the liquefied food is temporarily stored in the bag and does not occur digestion, proteolytic enzymes because this area is not secreted from the intestinal tract (Campobasso et al., 2005). Generally, nuclear DNA regions of, Short Tandem Repeats (STR) (Butler, 2001) and in particular two hypervariable regions (HVI and HVII) within the control region, non-coding or D-loop of the mitochondrial DNA (Lutz et al. 1998) are used to obtain human DNA profile.

The STR regions autosomal and sex chromosome are widely used by forensic science and can be applied to the analysis of the intestinal contents larvae dipterae. Additionally, in situations where regions of STR can not be typed due to the degree of DNA degradation, analysis of the SNP is an alternative because of its sensitivity (Kondacki et al., 2009).

Sometimes, with a low quantity and quality of DNA obtained through the analysis of the intestinal contents, the combination of techniques STR nuclear, mitochondrial DNA (Li et al., 2011) and SNP is necessary to increase the effectiveness of the results.

Wells and Stevens (2008) reported that with the improved constantly searches for forensic DNA, the ability to reliably characterize a sample in low quantity and quality removed from the previously inadequate insects content might be feasible in the future.

Today we know that the reality of getting these human DNA profiles arising from the larvae dipterae is a reliable tool and can be safely used by professionals in the forensic field. With advances in research involving entomogenetics and detailed studies of the digestive processes scavengers dipterous larvae, one can expect increasingly sensitive and specific techniques for obtaining human DNA profiles obtained in this group of insects.

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5.2 – Artigo 2

Insect Fauna Recovered in Putrefying Corpses at the Institute of Legal Medicine, Pernambuco, Brazil.

Periódico: Entomological Science (submetido)

Insect Fauna Recovered in Putrefying Corpses at the Institute of Legal Medicine, Pernambuco, Brazil.

Tatiana C. Oliveira[1,3]*, Arlene B.R. Santos[2], Kaynara C.N. Rabêlo[1,3], Sandra M. Santos[3], Sergio Crovella[1].

1 Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, Cidade Universitária, Recife, Pernambuco, Brazil.

2 Laboratório de Entomologia Forense, Área de Entomologia, Departamento de Biologia, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, Dois Irmãos, Recife, Pernambuco, Brazil.

3 Laboratório de Perícia e Pesquisa em Genética Forense, Secretaria de Defesa Social de Pernambuco, Estrada da Batalha, Prazeres, Jaboatão dos Guararapes, Pernambuco, Brazil.

***Corresponding author**
Tatiana Costa de Oliveira
E-mail: ttattian@yahoo.com.br.
Address: Rua André Dias Figueiredo, 120, ap. 102, Barro, Recife, Pernambuco, Brazil., CEP 50780-080.
Tel: +55-81-3251.0584 / +55-81-98726.2637. Fax: +55-81-2126.8485

ABSTRACT

Studies involving the main insect orders, such as Diptera and Coleoptera, are strongly assisting in criminal investigations. This study aimed to deepen knowledge about the insects associated with putrefying bodies at the Institute of Legal Medicine Antonio Persivo Cunha (IMLAPC/PE). Individuals collected at IMLAPC/PE were taken to the Forensic Entomology Laboratory of the Universidade Federal Rural de Pernambuco (LEF/UFRPE). Adults, packed in glass jars containing cotton soaked in chloroform, and immature in plastic pots of 70mL for further creation. An immature aliquot was placed in glass jars containing alcohol 70% by reference. The specimens of Order Diptera were created in bovine ground meat and the Order Coleoptera in the same type of substrate, but dry, until the emergence of adults. After emergence, the individuals were identified and stood up specimens of *Chrysomya albiceps* (Diptera: Calliphoridae) and *Dermestes maculatus* (Coleoptera: Dermestidae). Both species are considered of great importance for forensic science because of its abundance in human cadavers. This paper aims to contribute to the information related to colonization of human corpses for necrophagous insects and enhance existing data on the cadaverous fauna in this region.

Keywords: Forensic Entomology, Diptera, Coleoptera, Decomposition

INTRODUCTION

The biological decomposition process is started by the action of various microorganisms and continuity arise sarcosaprophagous arthropods groups which accelerate this process (Nuorteva, 1977). Two large orders of insects are involved in human body decomposition, the Order Diptera and the Order Coleoptera (Souza & Linhares, 1997) and their representatives are the most used as tools to provide data to assist in criminal investigations.

The main use of these insects is to determine the Post-Mortem Interval (Bornemissza, 1957; Schroeder et al., 2003), however, it can also be applied to studies dealing with removing bodies from a crime scene, obtaining human DNA taken from the gastrointestinal tract, toxicological analysis, among others (Goff & Lord, 2001).

The species of insects used for these purposes, have preferences for specific stages of decomposition (Campobasso et al., 2001) and vary according to the region thus climates, biomass and other environmental factors affecting

the distribution of the species (Anderson, 1997; Amendt et al., 2000; Grassberger & Reiter, 2002).

Thus, studies on insect fauna existing in specific regions need to be performed frequently, so that the entomological community that region is known with a certain accuracy. There are several types of surveys that can be performed as the use of specific traps, animal modeling in predetermined regions and collecting directly in decomposed bodies.

This last activity is very important because not all insects collected in the environment participate actively in the cadaverous colonization (Oliveira & Vasconcelos, 2010). Brazil has advanced enough in relation to insect studies associated with dead bodies, but the number of studies and reports is still small compared to the size of the land area and the number of species that can be linked to the process of decomposition.

The state of Pernambuco, northeastern Brazil, has few studies covering the issue, but in return has a high number of crimes of murder that could be used this tool to assist in the investigation.

So, this study aimed to analyze and identify insect specimens associated with decaying corpses collected by the Institute of Legal Medicine of Pernambuco (IMLAPC/PE) to contribute to the knowledge of the cadaverous insect fauna in the region.

MATERIALS AND METHODS

Material Collection

Four bodies were analyzed, all males, collected at the Institute of Legal Medicine Atônio Persivo Cunha (IMLAPC / PE). The material was collected with the help of histological tweezers, packed in plastic pots of 70mL and sent to the Forensic Entomology Laboratory at the Universidade Federal Rural de Pernambuco (LEF/UFRPE) for creation to adult emergency. A reference sample material collected was stored in alcohol 70% for later evaluation of larval instar and taxonomy. Adults were placed in vials containing cotton soaked in chloroform and after death, stored at 4°C for retrofitting and identification.

Maintenance of Immature

The dipteran larvae alive have been sent to the laboratory in 250mL jars containing sawdust as pupariation substrate and beef amount nutrition for immature in a 1:1 g/larvae. The pots were covered with fabric pieces, and kept at room temperature to simulate the temperature development in place of oviposition.

The immatures of the Order Coleoptera were kept in plastic pots of 2L containing sawdust as substrate and dried meat beef. The pots were also covered with fabric and kept at room temperature. The maximum and minimum temperatures and relative humidity were measured daily through thermohygrometer.

Identification of Specimens

With the emergence of adults, the assembly of the subjects was performed with the aid of pin n. 29, dried in an oven at 50°C for 24 hours and subjected to the identification of species and family levels, according to the entomological keys: Mello (2003); Triplehorn & Johnson (2007); Carvalho & Mello-Patiu (2008).

RESULTS AND DISCUSSION

We analyzed four corpses putrefying, all male, to the immature collections at the Institute of Legal Medicine Antônio Persivo Cunha (IMLAPC/PE). We established the pattern of decomposition as described in the literature for forensic medicine, and dividing the destructive decomposition, call decay, in four phases (França, 2004).

Chromatic or coloring period, is characterized by the appearance of a green stain on the skin; emphysema or gaseous period is characterized by gases which distend the organs and infiltrate the tissues; coliquative period or reduction of tissue, is characterized by the disintegration of the body structure;

skeletization period occurs with the destruction of the last ligaments and tendons, leaving exposed bones.

Two of the individuals analyzed were in a state of skeletization, one for gaseous and one in coliquativo state of decomposition. Two of these bodies had larvae in all stages of development (1st, 2nd and 3rd instars) while the corpse in a gaseous state had numerous ovipositions and newly hatched larvae, and one in late skeletization presented third instar larvae.

The first case was a male subject in a state of complete skeletization, in which there was a large concentration of larvae in the skull region (Figure 1). A lot of pupae of flies found inside the skull indicated that other generations this Order had already emerged. Larvae, pupae of newly emerged flies belonging to the family Calliphoridae, and Fanniidae and Stratiomyidae larvae were collected. After seven days of creation, began to emerge exemplary *Chrysomya albiceps* (Wiedemann, 1819), and with eight days specimens *Chrysomya megacephala* (Fabricius, 1794). On the ninth day of creation, individuals *of Fannia pusio* (Wiedemann, 1830) completing the cycle. In addition, adult beetles, *Omorgus suberosus* (Fabricius, 1775) (Trogidae), *Omalodes* sp. (Histeridae), *Dermestes maculatus* (De Geer, 1774) (Dermestidae), and larvae of three morphospecies unidentified, were present in the body. Copies of *Euborellia annulipes* (Lucas, 1847) (Dermaptera: Anisolabididae) were also collected. Representatives of Stratiomyidae Family were identified as belonging to specie *Hermetia illucens* (Linnaeus, 1758).

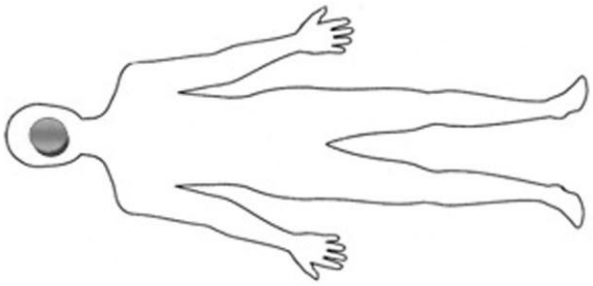

Figure 1: Scheme indicative the collection local of specimens in case 1.

The second case is an individual, also male, in coliquative phase of decomposition which had lot of immature in various stages of development spread throughout the body (Figure 2). The areas of highest concentration of

larvae masses were the cephalic region and the chest. After 12 days of creation emerged specimens of the species *Lucilia eximia* (Wiedemann, 1819) and *Chrysomya albiceps.* After 29 days of creation, specimens of *Oxysarcodexia* sp. (Diptera: Sarcophagidae) emerged.

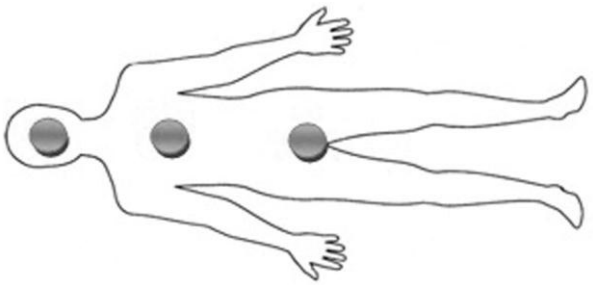

Figure 2: Scheme indicative the collection local of specimens in case 2.

The third case also features a male subject in a state of skeletization. Larvae were collected belonging to Diptera and Coleoptera, which were distributed throughout the body (Figure 3). The number of larvae was reduced due to the advanced state of body's decomposition and the degradation of teh majority of the soft tissue. Seven days after breeding individuals of the species *Chrysomya albiceps* emerged and ten days copies of *Hemilucilia segmentaria* (Fabricius, 1805). *Dermestes maculatus* (Coleoptera: Dermestidae) larvae were also placed in plastic pots containing sawdust and dried beef could initiate a creation.

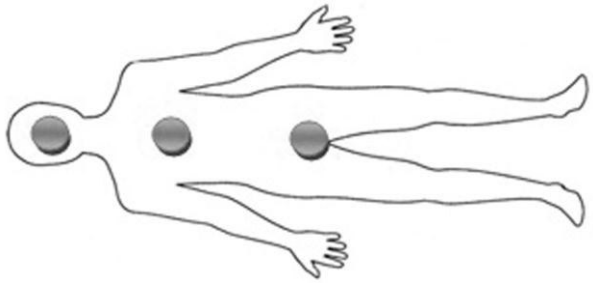

Figure 3: Scheme indicative the collection local of specimens in case 3.

The fourth and final assessed case concerns a male person in a gaseous state of decomposition. In this case the few larvae that had hatched were in first

instar and the vast majority of the body was filled with abundant oviposition (Figure 4). In this case, the oviposition were sent to the laboratory and specimen *Chrysomya albiceps* emerged after 12 days of creation.

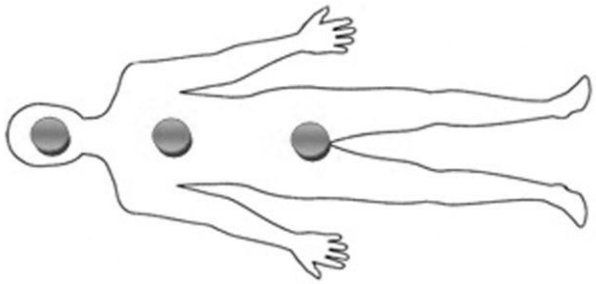

Figure 4: Scheme indicative the collection local of specimens in case 4

The first case, belonging to an individual in a state of skeletization, was the one with the highest number of species found. Although not having more organs and soft tissues, the body was attractive to individuals of the Order Diptera, mostly concentrated inside the. The literature does not show many cases similar to this, and despite the late stage of decomposition, we observed a number of species present in the body. Table 1 shows the species collected in each of the carcasses analyzed.

Table 1: Species collected in the corpses examined in Institute of Legal Medicine Antonio Persivo Cunha (IMLAPC).

Case / Phase Decomposition	Case 1 Skeletization	Case 2 Coliquative	Case 3 Skeletization	Case 4 Gaseous
Chrysomya albiceps (Wiedemann, 1819)	X	X	X	X
Chrysomya megacephala (Fabricius, 1794)	X			
Lucilia eximia (Wiedemann, 1819)		X		
Hemilucilia segmentaria (Fabricius, 1805)			X	
Fannia pusio (Wiedemann, 1830)	X			
Oxysarcodexia sp.		X		
Hermetia illucens (Linnaeus, 1758)	X			
Dermestes macullatus (De Geer, 1774)	X		X	
Omorgus suberosus (Fabricius, 1775)	X			
Omalodes sp.				
Morfoespécie 1	X			
Morfoespécie 2	X			
Morfoespécie 3	X			
Euborellia annulipes (Lucas, 1847).	X			

Specimens belonging to four species of family Calliphoridae: *Chrysomya albiceps*, *Chrysomya megacephala*, *Lucilia eximia* e *Hemilucilia segmentaria* have been found. The Calliphoridae family is considered one of the most important in forensic entomology. They are often the first to arrive and colonize corpses and rotting carcasses. The species of the genus *Chrysomya* are

important for being cosmopolitan and ease of adaptation to new environments (Guimarães et al., 1978, Oliveira-Costa, 2012).

The species *C. albiceps* and *C. megacephala* collected in the corpses, are exotic, being introduced in Brazil around the 1970s (Guimarães et al., 1978). After this introduction, they quickly adapted to our climate, spreading across all regions, competing and standing out over our native species (Dear, 1985; Grassberger et al. 2003).

The species *L. eximia* has saprophagous habit larval and has medical veterinary importance to be mechanical vector of several pathogens (Moretti & Thyssen, 2006). This species is considered neotropical and of forensic relevance in Brazil due to the large number of settlements detected in cadavers (Souza & Keppler, 2009).

H. segmentaria is an abundant species in natural environments, being virtually absent in urban areas. It is of great importance for forensic entomology, primarily as colonization location indicator (Thyssen & Linhares, 2007). His creation in the laboratory is considered rare, since it has high specificity for habitats.

These four species, *C.albiceps* and *L. eximia* showed better adaptation to captive breeding, reaching the sixth and fifth generations respectively. As previously mentioned, the great adaptation of the species to our climate, usually *C. albiceps* is the most abundant in cases of cadaveric colonization, the most used to estimate the postmortem interval. This can be confirmed in cases arising from the IMLAPC/PE, where this species was present in all of them and the only collected if four.

Representatives of Fanniidae family are commonly found associated with man-modified environments and use decaying organic matter to create their larvae (Marchiori & Prado, 1999). Previous work describe the presence of *Fannia pusio* of individuals associated with human corpses rotting in the region (Oliveira & Vasconcelos, 2010).

The species *Hermetia illucens* it is characterized by develop in decaying organic matter (plant bagasse, animal droppings), found in decomposing corpses (Ferrari et al., 2009). Pujol-luz et al. (2008), individuals of that species used to make the estimation of postmortem interval in a murder case.

The Oxysarcodexia genus has many species and wide distribution in Brazil, often described as dung, and sometimes predators (Silva & Mello-Patiu, 2009). Species of Sarcophagidae family, which gender is inserted, are quite common in corpses in the most active phases of decomposition, so we can associate its presence in the body coliquative phase. For individuals of Order Coleoptera, their presence in cases of corpses in skeletization and the absence in the other phases, strengthens their preference for the later stages of decomposition. Kulshrestha and Satpathy (2001) pointed out that when a corpse is already in the final stages of skeletization, a second very important group, after the flies, becomes another tool to calculate the postmortem interval, the Order Coleoptera.

The species *Dermestes maculatus* has been found in the two bodies in skeletization phase and their larvae were directed to creation. This species is considered of great forensic importance, being found in the later stages of decomposition and being responsible for cleaning the bones (Souza & Linhares, 1997; Carvalho & Linhares, 2001).

The larvae of *D. maculatus* are commonly found in corpses of advanced decomposition, they feed on and remain on dry skin after most other groups of adult beetles leave for other sources of food and mating (Von Hoermman et al., 2012).

The species *Omorgus suberosus* belonging to the Trogidae Family, is known, along with the Dermestidae family, guild by its well established as keratin consumers are drawn in a larger number for animals, such as birds and mammals, than for human corpses (Strümpher et al., 2014).

The species of the genus Omalodes, are commonly associated with decomposition of fruits and plants, also feeding on larvae and eggs of Diptera (Dégallier & Gomy, 1983; Mise et al., 2010). But studies such as Mise et al. (2010) and Almeida et al. (2015) report the presence of species of this genus present in decaying carcasses.

Individuals of the species *Euborellia annulipes* (Dermaptera) present omnivorous habits and may be associated with stored products or predatory attacking other species of insects (Neiswander, 1944; Silva et al. 2009). There are several accounts of his association with decaying animals (Braack, 1987; Goff et al., 1986).

The constant analysis of real cases involving forensic entomology and description of associated fauna, are of immense relevance to studies of the area and the constant complementation of data that can be incorporated into the records of the region.

Expected to always being the advance in studies involving the association of insects, mainly necrophagous, to cases of decomposition of human bodies. This study aims to contribute with more information for forensic entomology.

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The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) for the financial support. The Secretaria de Defesa Social de Pernambuco. Dr. João Batista Montenegro with the support collections in the Institute of Legal Medicine.

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5.3 – Artigo 3

Human autosomal DNA and X chromosome STR profiles obtained from *Chrysomya albiceps* (Diptera: Calliphoridae) larvae used as a biological trace

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Periódico: Genetics and Molecular Research (Aceito)

Human autosomal DNA and X chromosome STR profiles obtained from *Chrysomya albiceps* (Diptera: Calliphoridae) larvae used as a biological trace

Running Title: *C. albiceps* larvae as a biological trace for human DNA

T.C. Oliveira[1,3]*, A.B.R. Santos[2], K.C.N. Rabelo[1,3], C.A. Souza[3], S.M. Santos[3] and S. Crovella[1]

[1]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, Cidade Universitária, Recife, Pernambuco, Brazil.

[2]Laboratório de Entomologia Forense, Área de Entomologia, Departamento de Biologia, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, Dois Irmãos, Recife, Pernambuco, Brazil.

[3]Laboratório de Perícia e Pesquisa em Genética Forense, Secretaria de Defesa Social de Pernambuco, Estrada da Batalha, Prazeres, Jaboatão dos Guararapes, Pernambuco, Brazil.

***Corresponding author**

Tatiana Costa de Oliveira

E-mail: ttattian@yahoo.com.br.

Address: Rua André Dias Figueiredo, 120, ap. 102, Barro, Recife, Pernambuco, Brazil, CEP 50780-080.

Tel: +55-81-3251.0584 / +55-81-98726.2637. Fax: +55-81-2126.8485

ABSTRACT.

The use of insects to answer questions in criminal investigations, as well as the use of a combination of forensic genetics techniques to obtain human DNA from these organisms, especially necrophagous dipterians, has gained ground in recent decades among researchers and professionals in this area. The objective of our study was to evaluate and compare two methods of human DNA extraction, commonly used for forensic samples, to obtain human autosomal DNA and X chromosome short tandem repeat (STR) profiles from the digestive tract of *Chrysomya albiceps* (Diptera: Calliphoridae) larvae. Immature specimens were collected from corpses at the Institute of Forensic Medicine of Pernambuco and raised in bovine ground meat to allow stabilization of the colony. Groups of larvae in the third instar were provided with bovine ground meat plus human blood for 48 h, dissected, and then subjected to DNA extraction. DNA was extracted using two methods: a DNA IQ™ kit and a phenol-chloroform method. Genomic DNA was amplified using AmpFℓSTR® Identifiler® Plus PCR and Argus-X-12® kits, and samples were sequenced to determine if the two extraction techniques generated reliable profiles that were compatible with a reference sample. The existence of comparable profiles from both techniques demonstrates the usefulness of dipteran larvae for obtaining human DNA from corpses, which can be further used to correlate genetic profiles in a crime scene when other traces are not available. However, several variables still require revision; thus, the technique should be further investigated for its validity, security, and, in particular, its reproducibility.

Key words: Forensic Genetics; Forensic Entomology; Dipterians; Short Tandem Repeats; Uniparental Markers

INTRODUCTION

Forensic entomology, the study of insects associated with forensic procedures, aims to gather information and traces that can assist an investigation, demonstrating the importance of both science and legal processes (Thyssen, 2011). Among the various uses of insects in

investigations, entomogenetics combines forensic genetic techniques and the versatility of entomology.

Among the various techniques currently used for individualization of people in a forensic context, DNA is notable, especially in crime scenes where various types of biological materials are commonly found, often in minute quantities, masked, mixed, degraded, contaminated, and decomposing (Ziętkiewicz et al., 2012). In the case of crime scenes, all traces left by the perpetrator or the victim may be essential for clarification of the facts, regardless of the type, quality, or quantity of material.

Wells et al. (2001) identified a number of situations in which food content analysis of the larval gut may be used in forensic investigations. Human DNA was found in blood from the abdomen of mosquitoes up to 26 h after ingestion, demonstrating that DNA isolated from mosquitoes is qualitatively and quantitatively sufficient for DNA typing (Kreike and Kampfer, 1999).

Human DNA isolated and amplified from pubic lice-blood can be helpful in an investigation aimed at identifying individuals involved in certain cases of sexual violence because of transmission of insects from the perpetrator to the victim during the act (Campobasso et al., 2005). Human mitochondrial DNA (mtDNA) has also been obtained from beetle larvae recovered from bones exposed to the environment for several months (Linville et al., 2004).

Recently, the assessment of cases and simulation studies based on the analysis of short tandem repeats (STR) in human DNA extracted from the intestinal contents of fly larvae has shown that flies can provide molecular evidence for identifying offenders and victims (Kester et al., 2010).

In Brazil, few studies involving entomology together with genetics have been conducted so far for forensic purposes. The current research aims to contribute to the advancement of these studies, enabling the use of new tools to increase the degree of accuracy of techniques used during criminal investigations. The union of entomology with forensic genetics can bring greater

effectiveness to evidence generated during criminal investigations. However, many gaps remain to be filled, and data generated in the academic environment should be applied to instances responsible in the conduct of the criminal process.

The state of Pernambuco in Brazil has a high rate of homicides and crimes, especially in its capital, Recife, and many of these have no apparent solution. High crime, associated with a lack of empirical studies in the state, underscores the importance of studying, in depth, a new tool that can support investigations conducted by the local scientific police.

This study aims to help fill some of these gaps and also support the research data previously generated, which could help to improve methods for the analysis and validation of evidence in criminal cases.

MATERIAL and METHODS

Dipterians with immature bodies were collected from corpses at the Forensic Medicine Institute Antonio Persivo Cunha of Pernambuco (IMLAPC/PE) in order to establish a colony of flies for the experiment. The material was collected with the help of histological tweezers, packed in 70-mL pots, and sent to the Forensic Entomology Laboratory at the Federal Rural University of Pernambuco (LEF/UFRPE) for colony creation. A sample of reference material was collected and stored in 70% alcohol for later evaluation of larval instars and taxonomy.

Immature larvae were brought to the laboratory alive in 250-mL plastic pots containing sawdust as puparian substrate and bovine ground meat for immature nutrition at a ratio of 1:1 g/larvae. The pots were covered with fabric pieces and maintained at room temperature, with an average of 28°C (±2°C), to simulate temperature development in place of oviposition. The maximum and minimum temperatures and relative humidity were measured daily using a thermohygrometer. When adults emerged, they were assembled with the aid of pin number 29, dried in an oven at 50°C for 24 h, and subjected to identification

to species and family levels in accordance with an entomological key by Carvalho and Mello-Patiu (2008).

The emerging adults were maintained in cages (20 × 20 × 50 cm; length × width × height), containing a fabric sleeve for colony maintenance. The subjects were fed with two solutions. First, an aqueous solution containing sugar and water (1 g/10 mL) and second with powdered milk and powdered yeast (2 g/2 g in 5 mL water). In addition, bovine ground meat was used as a substrate for female oviposition. The whole setting was maintained at room temperature, around 28ºC (±2°C), which was monitored daily with the aid of a thermohygrometer. Following oviposition in the bovine ground meat, the substrate was transferred to plastic pots containing sawdust to allow larval development to be completed. Following development, second-generation individuals were used in the experiments.

For simulation purposes, prior to feeding on bovine ground meat without any sources of human DNA, larvae were provided with bovine ground meat plus human blood. Blood material from a female person was used for analysis of autosomal DNA and X chromosome STR loci, at a ratio of 20 g bovine ground meat to 1 mL fresh human blood for each group of 20 larvae. The specimens were selected at the third instar larvae stage and were allowed to feed on the diet for 48 h.

The dissection of immature larvae was carried out following the recommendations by Kondakci et al. (2009). Immature larvae were removed from the substrate and placed in 70% alcohol for 5 min to remove impurities that may be on the surface of the individual. Subsequently, the dissection was carried out on an exemplary glass slide by extracting the cephalic part and the entire cephalopharyngeal skeleton using histological scalpel and tweezers. The remainder of the larva was squeezed and extracted viscera were placed individually into 1.5-mL microtubes containing 1 mL distilled water. After dissection, the samples were stored at 4°C. Each larva corresponds to a single sample being tested.

DNA was extracted from dissected material using both the DNA IQ™ kit (Promega, Madison, WI, USA) and an organic extraction protocol using phenol-chloroform. For the reference sample, which consisted of a swab of oral mucosa taken from the same source as the human blood samples, DNA was extracted using only the DNA IQ™ kit (Promega). Reference samples are always rich in DNA; therefore, there was no need to compare extraction techniques for these samples.

DNA extraction using the DNA IQ™ kit (Promega) was undertaken following the manufacturer protocol, which is commonly used in the routine procedures of the Expertise and Research Laboratory for Forensic Genetics of the Social Defense Secretariat of Pernambuco (LPPGF/SDS-PE). Organic extraction was performed based on the protocol described by DiZinno et al. (2002) using phenol, chloroform, and isoamyl alcohol, and filtering with Microcon™.

Autosomal DNA was quantified using the Quantifiler® Duo DNA quantification kit (Applied Biosystems, Foster City, CA, USA) and a real-time PCR 7500 system. The quality of DNA was also assessed for a number of samples using a Nanodrop® ND-1000.

Extracted DNA was amplified using a commercial multiplex kit for the study of autosomal STRs, the AmpFℓSTR® Identifiler® Plus PCR kit (Applied Biosystems), following the manufacturer protocol.

This kit generates profiles for 15 autosomal loci and amelogenin STRs to enable sexual differentiation (D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818, FGA, and Amelogenin). In order to obtain the X chromosome STR loci, we used the Argus-X-12® kit (Qiagen GmbH, Hilden, Germany), which has 12 markers and amelogenin (DXS7132, DXS7423, DXS8378, DXS10074, DXS10079, DXS10101, DXS10103, DXS10134, DXS10135, DXS10146, DXS10148, and HPRTB). Fragments were analyzed by capillary electrophoresis using a DNA genetic analyzer (sequencer), the ABI Prism 3500 model, following the manufacturer protocol. All cited material is commonly used by the Forensic Genetics Laboratories worldwide.

Methodology success indicators were used to verify the quantity and quality of DNA obtained by amplification of nuclear DNA regions for forensic analysis. The results were analyzed using GeneMapper® ID-X software (Applied Biosystems, 2009), to score the alleles in electropherograms produced by the sequencer. The alleles generated in the electropherograms were passed through the statistical analysis software Patcan V.1.2 (Riancho and Zarrabeitia, 2003) to perform comparison of trace samples. The statistic was performed as described by Buckleton et al. (2005).

RESULTS

Insects collected from corpses at the IMLAPC/PE and sent to the LEF/UFRPE were monitored until stabilization of the colonies and identification of specimens. Among the species collected, we selected *Chrysomya albiceps* (Wiedmann, 1819; Diptera: Calliphoridae) as a model.

Groups of 20 third instar larvae of *C. albiceps*, left in bovine ground meat and human blood for a period of 48 h to enable stability of the colony, were dissected and used to test two DNA extraction methods. This period of 48 h was selected to ensure higher levels of larval activity with the same diet: larvae were not killed prior to dissection and when viewed following dissection, the larval gut was an intense red color.

Larval dissection was based on the methodology described by Kondakci et al. (2009), which was used throughout the larvae visceral content. We adopted this technique for simplicity and reproducibility, so that it can be used by forensic professionals and academics. Since many criminal experts working with forensic genetics in Brazil do not have specific knowledge of entomology, this technique is more accessible than the gut larval dissection itself.

Following dissection of the larvae, DNA extraction was carried out. Groups of ten larvae were subjected to two extraction methods commonly used in a forensic context: the DNA IQ™ kit (Promega) and an organic extraction with phenol-chloroform.

The two extraction techniques were successful in obtaining human autosomal DNA from the larvae that was compatible with a reference sample, generating full profiles that matched the reference buccal swab mouth sample (Figure 1). Our results show complete profiles of human STRs and this only occurs for a short period during degradation of the material, typically within 48 h. This means that, within the first 48 h of death, full DNA profiles can be obtained from larvae.

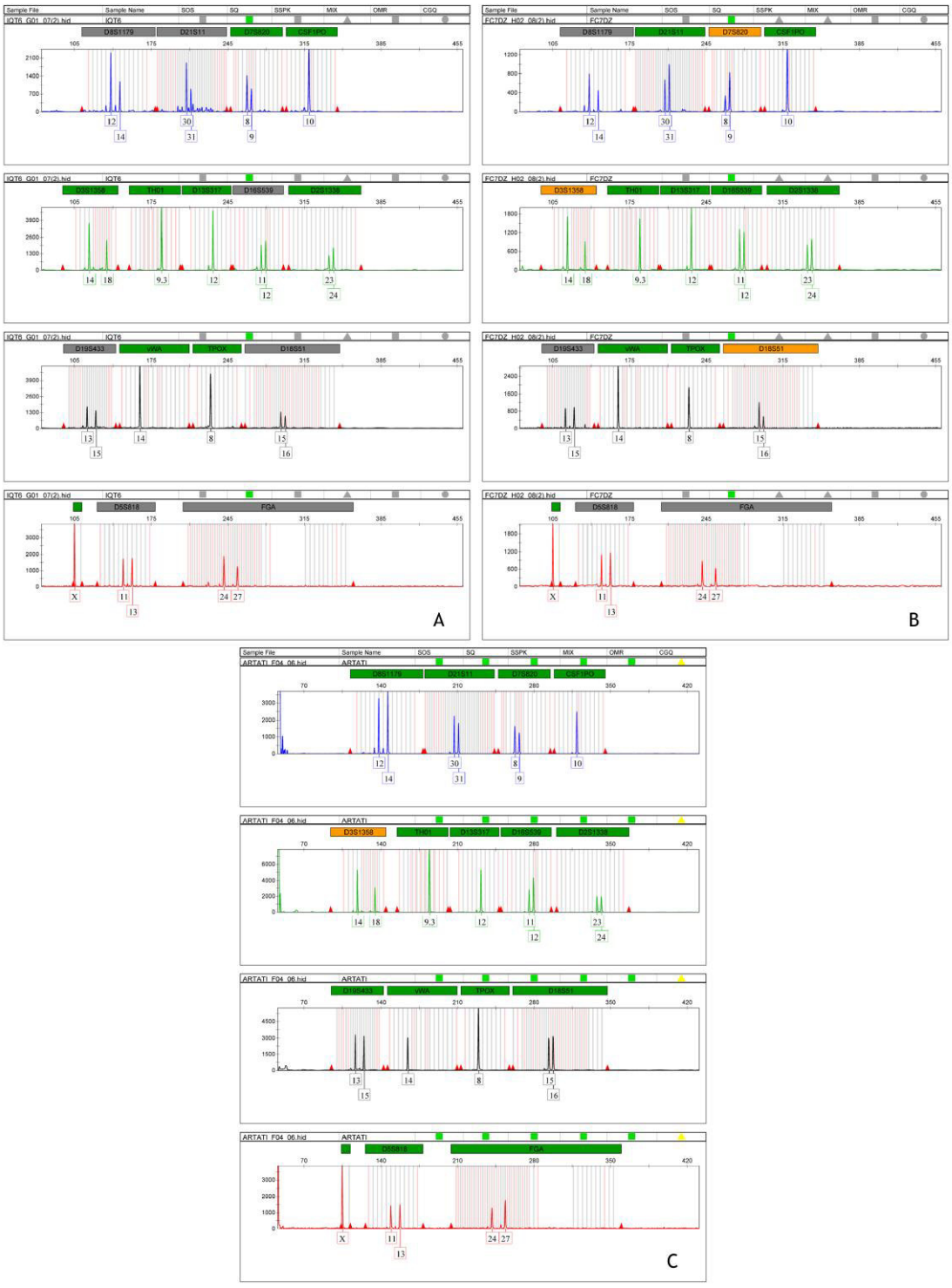

Figure 1: Comparison of autosomal DNA and X chromosome short tandem repeat (STR) profiles from (A) human DNA from *Chrysomya albiceps* larvae extracted using a DNA IQ™ kit (Promega); (B) human DNA from *C. albiceps* larvae extracted using a phenol-chloroform method; and (C) human DNA obtained from an oral buccal swab used as a reference sample.

In regards to the DNA IQ ™ kit, although it is more expensive, it has low toxicity and a relative sensitivity due to magnetic particles made of silica that

can attract and deliver the DNA contained in the sample. It is also used for small DNA samples, such as the subungual ones.

The quality of DNA samples in the current study was assessed using a Nanodrop® ND-1000 and the quantity of DNA was measured using real-time PCR. The results obtained using the Nanodrop® showed large amounts of inhibitory substances in all cases (Table 1). These substances are probably derived from lipids and other elements contained in the gut of the larvae.

Table 1: Quality of human DNA samples extracted from the gut contents of *Chrysomya albiceps* assessed using a Nanodrop® ND-1000. * denotes samples not assessed due to low quantities of material.

Sample	230nm	260nm	280nm	260/230	260/280
IQT1	129.2	20.45	11.7	0.15	1.74
IQT2	81.8	17.6	9.6	0.21	1.83
IQT3	470.35	23.3	13.1	0.04	1.77
IQT4	220.45	19.5	10.6	0.08	1.83
IQT5	258.15	20.55	10.9	0.07	1.88
*FC6DZ	------	------	------	-------	-------
*FC7DZ	------	------	------	-------	-------
FC8DZ	397.2	531.65	317.15	1.33	1.67
FC9DZ	167.2	246.9	153.2	1.47	1.61
FC10DZ	233.65	339.5	204.65	1.45	1.65

All samples were also quantified by real-time PCR using the Quantifiler® Duo DNA Quantification kit (Applied Biosystems). This kit is highly specific for human DNA and detects degraded and single chain samples. Real-time PCR revealed very low DNA amounts (and in most cases was not able to provide quantification) for the samples analyzed (Table 2).

Table 2: Quantity of human DNA in samples extracted from the gut contents of *Chrysomya albiceps*, determined using real-time PCR with a Quantifiler® Duo DNA Quantification kit (Applied Biosystems).

Sample	DNA Quantity (ng/µL)
IQT1	Undetermined
IQT2	0.0010
IQT3	Undetermined
IQT4	Undetermined
IQT5	Undetermined
FC6DZ	0.0030
FC7DZ	Undetermined
FC8DZ	Undetermined
FC9DZ	Undetermined
FC10DZ	Undetermined

Although the vast majority of samples quantified using real-time PCR had indeterminate results, we were able to obtain full autosomal STRs using sequencing. The results of genotyping demonstrated that for larvae of *C. albiceps* used in the experiment with the AmpFℓSTR® Identifiler® Plus kit (Applied Biosystems) the results were reliable. The AmpFℓSTR® Identifiler® kit has been successfully used in other studies that also analyzed human DNA extracted from insects and achieved a good level of amplification (Wang et al., 2009; Li et al., 2011; De Lourdes Chávez-Briones et al., 2013).

Statistical analysis, using the rate for the population of the state of Pernambuco, showed that the profiles obtained by analyzing the intestinal contents of the larvae of *C. albiceps* were 100% compatible with the reference sample taken through the buccal mucosa swab.

Comparison of the profiles obtained using the two DNA extraction methods showed major peaks for the majority of the loci amplified using DNA extracted with the commercial kit (Figure 2). This shows that, although more expensive, this kit may be used for samples with very limited effectiveness.

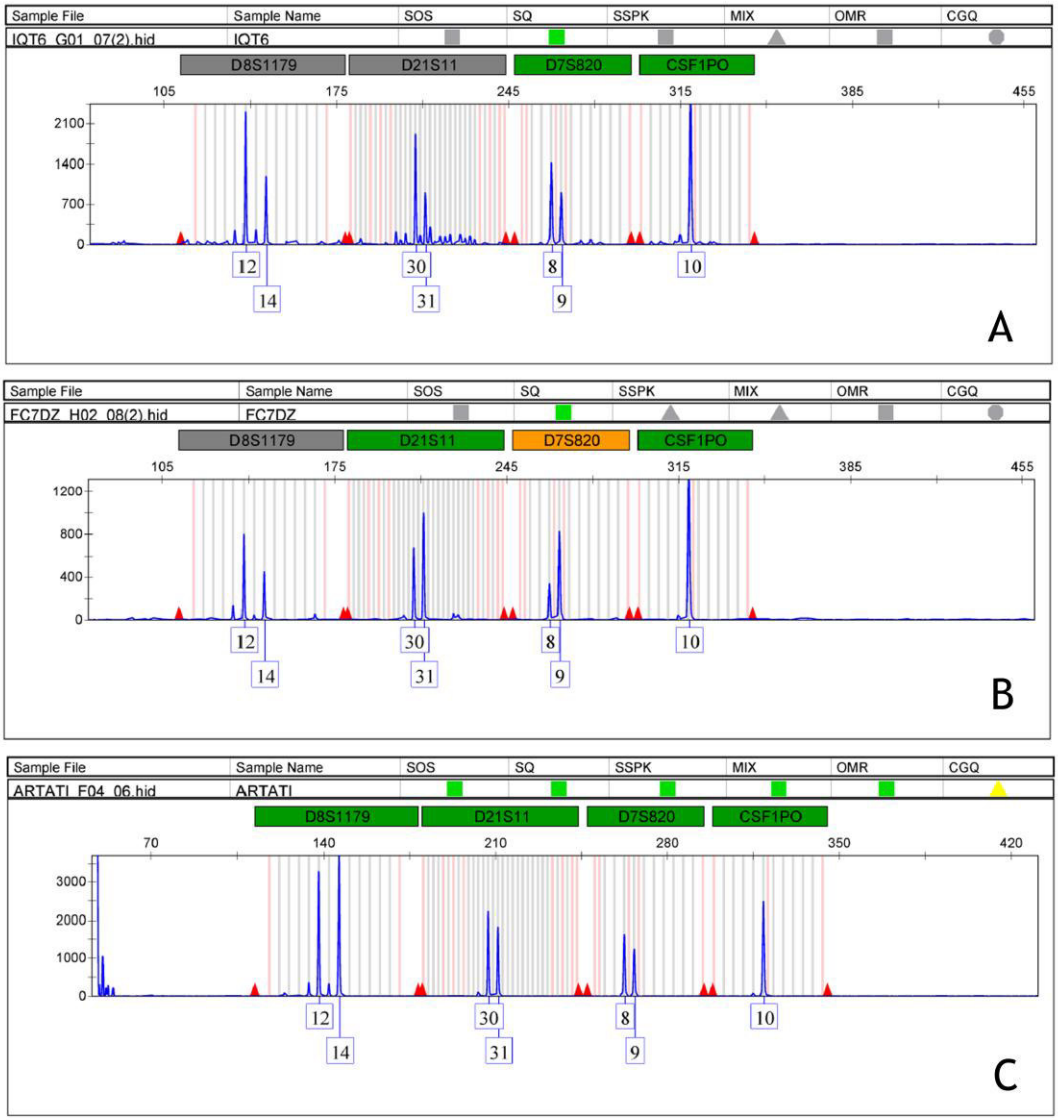

Figure 2: Comparison of peak height for loci D8S1179, D21S11, D7S820 and CSF1PO in (A) human DNA from *Chrysomya albiceps* larvae extracted using a DNA IQ™ kit (Promega); (B) human DNA from *C. albiceps* larvae extracted using a phenol-chloroform method; and (C) human DNA obtained from an oral buccal swab used as a reference sample.

With respect to obtaining loci related to the X chromosome, the samples generated full, reliable profiles, which were compatible with the reference sample (Figure 3).

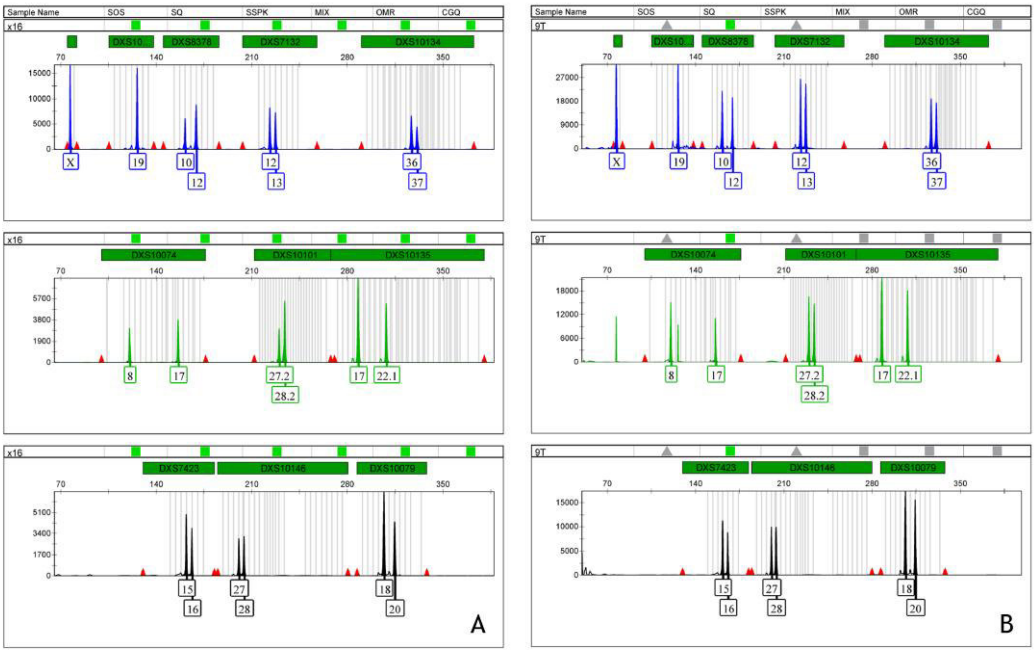

Figure 3: Comparison of X chromosome haplotypes from (A) human DNA obtained from an oral buccal swab used as a reference sample and (B) human DNA extracted from the gut contents of *Chrysomya albiceps* larvae.

DISCUSSION

We selected *Chrysomya albiceps* (Wiedmann, 1819; Diptera: Calliphoridae) as a model because of its great importance for forensic science in Brazil, based on its wide distribution and abundance in the Brazilian territory in the colonization of bodies. These factors make this species one of the most important to study and for application as a tool for investigations involving forensic entomology. Despite being an exotic species in Brazil, *C. albiceps* began its expansion across the country in the 1970s (Guimarães et al., 1978) and today it is the most commonly found species in studies of wildlife inventories, simulation studies, and real case studies, standing out in comparison to native species (Grassberger et al., 2003; Oliveira-Costa and Mello-Patiu, 2004; Arnaldos et al., 2005; Oliveira and Vasconcelos, 2010).

Studies suggest that larvae of the third instar actively feeding on a corpse can be considered a great source of human DNA (Campobasso et al., 2005). The experimental time used here was also selected to replicate the decomposition process, which in tropical countries is faster, thus, the larval feeding time was 48 h, enough to simulate a corpse between the gas phase and colliquative putrefaction.

The gut from the larvae of Diptera does not undergo the action of enzymes that may degrade food and consequently DNA (Hobson, 1931). For this reason, it was chosen by experts as the main source of human DNA during forensic analyses. However, while the larval gut is the best choice for DNA extraction, previous training is necessary in order to correctly dissect this region.

The literature on human DNA extracted from the larvae of muscoid flies usually yields results from mitochondrial DNA, while many autosomal STR profiles are only partially reported (Wells et al., 2001; Zehner et al., 2004; Campobasso et al., 2005; Wells and Stevens, 2008; Wang et al., 2009; Li et al., 2011). Mitochondrial DNA is used when sample degradation does not allow analysis of autosomal markers. This degradation process is also responsible for obtaining partial profiles.

The organic extraction method is considered by experts and researchers to be the most sensitive method for extracting DNA from forensic samples classified as challenging, e.g., bones. Despite its low cost and high efficiency, this technique is quite toxic, employing reagents such as phenol, chloroform, and isoamyl alcohol. Zehner et al. (2004) used the organic extraction method to obtain human DNA from larvae collected from cadavers, obtaining good results. As described by Carvalho et al. (2005), who compared the organic extraction method with commercial kits, it is the most effective method. The DNA IQ™ kit was used by Di Luise et al. (2008), who identified a high degree of variation between samples, although useful results were generated. Furthermore, De Lourdes Chavez-Briones et al. (2013) obtained a DNA profile from a crime

victim through larvae and were able to compare it with the profile of the alleged father, confirming the identity of the victim.

The AmpFℓSTR® Identifiler® kit has been successfully used in other studies that also analyzed human DNA extracted from insects and achieved a good level of amplification (Wang et al., 2009; Li et al., 2011; De Lourdes Chávez-Briones et al., 2013).

The X chromosome is important for forensic analysis as it increases the probability of connecting a genetic profile to a person. With autosomal markers added to the sex chromosome, the probability of individualization is increased.

In cases where autosomal markers cannot be used because of DNA degradation or other factors, generating incomplete or no profiles, the X chromosome becomes a guiding tool for the ongoing investigation.

With few exceptions, markers of the X chromosome are less powerful in the analysis of trace samples than autosomal markers (Toni et al., 2006). However, the identification of feminine characteristics in admixture with male samples, the X chromosome markers can be more efficient since the female alleles could only be fully included in the male haplotype if this were completely homozygous for loci analyzed (Szibor, 2007).

The assessment of female remains in a sex crime with a male suspect by typing the X chromosome has already been successfully carried out (Szibor et al., 2003; Szibor, 2007). The analysis of X chromosome markers from DNA samples derived from insects is still largely unexplored and only a few extraction protocols are available in the literature. The protocol used in this study for DNA extraction followed an earlier method used for autosomal STRs.

Wells and Stevens (2008) highlight the need for specific protocols aimed at recovering human DNA from insects, since any forensic expert can be, in the near future, required to perform this analysis. In order to meet this need of the professional forensic field, we designed experiments to generate reliable

protocols that pass the expert techniques required for effective human DNA analysis of samples obtained from insects to non-experts. Protocols that describe all necessary steps for the success of the technique, ranging from the correct way to collect, dissect, and store samples, through to extraction and amplification to achieve the desired genetic profiles.

The results to date show that human DNA extraction from the larvae of necrophagous dipterians can generate upright profiles, as well as other forensic context samples (blood, muscles, bones). Gut content analysis of these larvae is a new method in entomology and forensic genetics, and many of the technical limitations remain unexplored. Although there are several studies that address this issue, it is known that success in the technique depends on insect development and metabolism.

The metabolism of the digestive tract of forensic interest dipterans varies according to the family, genus, and species of the insect. In addition, climatic factors directly influence the digestion of specimens. In this way, the studies published and presented in other countries do not match the reality of the specimens found in Brazil, in particular in the north eastern part of the country where we operate (Amendt et al., 2000).The warm and humid climate, with higher temperatures throughout most of the year, causes a body to undergo rapid decomposition and, consequently, the power and digestion by necrophagous dipterans also increases (Oliveira-Costa and Mello-Patiu, 2004).

Analysis of the various extraction methods and amplification kits in the current study reveals that these types of sample cannot be treated in the same manner as those commonly used in criminal investigations. The gut contents of the larvae must first be treated by removing any substance, such as lipids and proteins, which interfere with DNA amplification.

From these experiments, it is expected to finalize and validate a protocol that details a step by step methodology that forensic professionals or scientist could follow to obtain reliable results from this type of sample and apply them to real cases. It is expected that the protocol can be followed both by experts in

genetics and entomology, or by a professional with little experience in either area. Ideally, a criminal expert should be able, based on the protocols, to conduct an expert analysis in entomogenetics without specialist knowledge of either disciplines.

Currently, published protocols that could be used for DNA extraction from necrophagous insect larvae are scarce and do not offer very detailed procedures. Moreover, they do not use species and temperature conditions similar to those experienced in Brazil. Therefore, it is necessary to deepen the techniques and protocols that can be used in Brazil to better enforceability of entomogenetics, reproducibly, which is applicable to different species that have similar growing conditions, with relative safety.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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5.4 – Artigo 4

Influence of storage methods in obtaining human DNA from *Chrysomya albiceps* (Diptera: Calliphoridae) larvae.

Periódico: Journal of Medical Entomology (Submetido)

Influence of storage methods in obtaining human DNA from *Chrysomya albiceps* (Diptera: Calliphoridae) larvae.

Tatiana Costa de Oliveira[1,2*], Carlos Antônio de Souza[2], Sandra Maria dos Santos[2], Kaynara Cecília Nery Rabêlo[1,2], Arlene Bezerra Rodrigues dos Santos[3], Sergio Crovella[1].

[1]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, Cidade Universitária, Recife, Pernambuco, Brazil, CO 50670-901.

[2]Laboratório de Perícia e Pesquisa em Genética Forense, Secretaria de Defesa Social de Pernambuco, Estrada da Batalha, Prazeres, Jaboatão dos Guararapes, Pernambuco, Brazil, CO 54315-010.

[3]Laboratório de Entomologia Forense, Área de Entomologia, Departamento de Biologia, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, Dois Irmãos, Recife, Pernambuco, Brazil, CO 52171-900.

Running head: Oliveira et al.: C. albiceps storage methods for obtaining human DNA.

***Corresponding author**
Tatiana Costa de Oliveira
E-mail: ttattian@yahoo.com.br.
Address: Rua André Dias Figueiredo, 120, ap. 102, Barro, Recife, Pernambuco, Brazil, CO 50780-080.
Tel: +55-81-3251.0584 / +55-81-98726.2637. Fax: +55-81-2126.8485

ABSTRACT

The use of the food contents of dipterous necrophagous has become a new tool for obtaining human DNA. Nevertheless, there is still deficient in the standardization of protocols for this type of technique. Thus, this study aimed to test storage media commonly used in fixing entomological samples and/or forensic and determine its interference for the analysis of DNA. For this, larvae in the third instar, *Chrysomya albiceps* (Diptera: Calliphoridae), were subjected to in vitro experiments where, for 48 hours were allowed for food source comprises bovine ground beef and human blood in the ratio 1: 1 gram by larva. After this time period, the larvae were dissected and larval crop was stored in 1.5 ml microtubes, using four methods, 70% ethanol, 95% ethanol, 4% formaldehyde and dry. After 24 hours of storage the samples were removed from the respective media and subjected to DNA extraction using phenol, chloroform and isoamyl alcohol. The analysis of autosomal STR markers was performed by amplification with the commercial kit PowerPlex® Fusion System. The amplified material was genotyped in capillary electrophoresis genetic analyzer ABI Prism 3500 model. The results show that the only way it was possible to generate full access to human DNA, was 4% formaldehyde. The rest of the media, there was degradation of samples, it was not possible to obtain an integrity profile. Thus, if there is the need for sample storage arising from muscoid flies, the 4% formaldehyde is an alternative to preservation of both the extrinsic characteristics as compared to the DNA. However, it is still necessary that further studies are developed for corroboration of the data.

Keywords: Forensic Entomology, Diptera, Forensic Genetics, STR markers.

INTRODUCTION

Insects have been used for applicability in the modern criminal context for several decades. These applicability, has been growing and improving as research and development techniques is advancing.

Although still using the insects that are associated with the cadaverous decomposition process primarily to determine the postmortem interval, obtaining human DNA from the intestinal contents of dipterae larvae is the newest tool of forensic entomology (Li et al. 2011).

The uses of human DNA profile obtained from the intestinal content larvae may be many, emphasizing the possibility of, for example, confirming whether a larva belongs even a corpse, in case there is more than one source nearby food, or yet, in an exchange of samples, one can confirm the link larva with crime and safeguard the chain of custody (Linville and Wells 2003).

It is common for the collection of entomological material to be used for the skill to be performed in distant location where it will be analyzed, making it necessary to use samples preservation methods (Oliveira et al. 2002). The preservation entomological test becomes even more important if the DNA analysis of the intestinal contents of muscoid dipterous larvae will be used, because some methods for the preservation of larvae for taxonomic tests, for example, may not be suitable for maintaining DNA intact (Linville et al. 2004).

Thus, preservation methods for Diptera larvae that will serve as human DNA profile source should be tested and standardized for the analysis of the food content of these insects is not impaired. The research on this type of methodology are still scarce in the scientific community, therefore, this study aimed to analyze different means of preservation of entomological samples commonly used in scientific research and demonstrate what are the most efficient methods for maintenance of organisms that will not threaten analysis human DNA profile contained in the intestinal contents of these insects.

MATERIALS AND METHODS

The experiment consists of simulation in vitro obtaining human DNA from the food content larvae dipterae. For this, larvae were used kept in laboratory rearing and human material known source, used as reference sample.

Muscoid flies larvae were collected at the Institute of Forensic Medicine Antonio Persivo Cunha (IMLAPC/PE) in a corpse in colliquative stage of decomposition and sent to the Forensic Entomology Laboratory at the Federal Rural University of Pernambuco (LEF/UFRPE) in plastic pots of 70mL, cages, for screening, creation and identification of specimens.

Part of the larvae was stored in 70% ethanol to serve as a future reference on instar and characteristics and size of the immature. The remainder was placed in 500mL plastic pots containing sawdust as pupariation substrate and minced meat beef in the ratio of 1:1 gram per larva, as a food source. The larvae were maintained at room temperature, on average 28°C (± 2°C) to simulate conditions similar to develop a crime scene. The maximum and

minimum temperatures as well as moisture from the place of rearing were measured daily.

After emergence of adults was killed with the use of chloroform and mounted with the aid of pin number 29. The specimens were left in an oven at 50°C for a period of 48 hours for dehydration and subsequent taxonomic identification. Identification to species level was carried out through the use of taxonomic key de Carvalho and Mello-Patiu (2008).

The rest of the emerged adults, were placed in screened cages 20x20x50cm in size (Length x Width x Height) for maintenance of new generations. The creation was also carried out at room temperature with daily measurement of temperature and humidity patterns. For nutrition were offered a mixture of sugar and water (1g/10mL) with powdered milk and powdered yeast (2g/2g in 5mL water). In addition, bovine ground meat was offered as a substrate for oviposition.

For initiation of the in vitro experiment, after the third generation of individuals, groups of 20 larvae entering third instar, were separated and put into plastic pots of 500 mL, containing sawdust as pupariation substrate and feeding minced meat beef base and human blood was offered in a ratio of 20g to 1mL, following standard nutrition 1:1 gram of meat per larva.

The larvae had no prior contact with any source of human DNA and were kept in bovine blood added ground beef for a period of 48 hours to simulate a tissue decomposition process in the initial stages of putrefaction. With the confirmation of supply by viewing the intense color of the digestive tract, the larvae were removed from the food and forwarded dissection.

The dissection was carried out following the pattern described by Kondracki et al. (2009), where a cut is made in the framework region cephalopharyngeal larvae and compression to remove the digestive tract. After the location of the crop, situated in the region of the foregut, it was removed and separately for the in vitro experiments.

Each larval crop belonging to one larva was considered single sample. The conversations were stored in 1.5mL microcentrifuge tubes and separated into categories according to the storage medium used. The groups were

categorized into: 70% ethanol, 95% ethanol, 4% formaldehyde and dry tube, and kept at room temperature averaging 28°C (± 2ºC).

After 24 hours of storage, crop larval submitted by phenol extraction technique, chloroform and isoamyl alcohol following the modified protocol DiZinno et al. (2002), with Microcon™ filtrate. The reference sample donor was extracted by DNA IQ™ Kit (Promega, Madison, WI, USA), following the manufacturer's recommendations.

The extracted DNA was amplified using the commercial kit PowerPlex® Fusion System (Promega) containing 24 genetic loci (D3S1358, D1S1656, D2S441, D10S1248, D13S317, D16S539, D18S51, D2S1338, CSF1PO, TH01, VWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, D22S1045, FGA, Penta E, Penta D, DYS391 and Amelogenin).

The analysis of amplified fragments was performed in capillary electrophoresis genetic analyzer (sequencer), model ABI Prism 3500 (Applied Biosystems, Foster City, CA, USA) following the recommendations of the said piece of equipment. All the material used is commonly adopted in forensic genetics laboratories around the world.

To analyze the success of the techniques applied, quality indicators and amount of amplified DNA were employed. The results were analyzed using the software GeneMapper® ID-X (Applied Biosystems 2009), determining the alleles produced the electropherogram by the sequencer.

Alleles presented in electropherograms underwent statistical analysis by Patcan V.1.2 software (Riancho and Zarrabeitia 2003), used to compare samples in traces of clashes. Statistical follows that described by Buckleton et al. (2005).

RESULTS AND DISCUSSION

Among the insects collected from corpses in the Forensic Medicine Institute Antonio Persivo Cunha (IMLAPC/PE), the most abundant species was the *Chrysomya albiceps* (Wiedmann 1819) (Diptera: Calliphoridae) which by its relevance to the Forensic Entomology and its wide distribution was selected as a model for this study.

These factors make C. albiceps is one of the main species used as a tool in criminal investigations. This species arrived in Brazil still in the 1970s (Guimarães et al. 1978) and its wide adaptability has a lot of real studies and uses, making it one of the most species well described in the literature (Grassberger et al. 2003, Oliveira-Costa and Mello-Patiu 2004, Arnaldos et al. 2005, Oliveira and Vasconcelos 2010).

The region of the digestive tract of *C. albiceps* immature chosen for DNA analysis was the larval crop. This region is known to store the food for a period of time until it is sent to the midgut. Although there are some enzymes present in the region, digestion itself occurs in the midgut (Hobson 1931).

The storage media chosen for the larval crop dissected samples follow the standards commonly used for storage insects and other invertebrates, 70% ethanol, 95% ethanol, 4% formaldehyde and dry storage would not have interference from any chemicals. After dissection, performed as recommended by Kondacki et al. (2009), the selected part to the molecular examinations were separated into four groups, following previously described.

After the first 24 hours of storage the samples were removed from the preservation medium and sent for molecular testing. The analyzes showed that in all the methods of preservation there was some sort of loss in the genetic profile generation. When a larva is dissected directly at the time of collection of the food source and the DNA extraction is then carried out and then obtaining the DNA profile is integrated in sufficient quality and quantity to generate a test with forensic applicability (Oliveira et al. 2016).

But over time, even amid the samples commonly used for preservation, they had loss of quality in obtaining the genetic profile, not generating upright profiles that could generate a test with effective forensic applicability. These data contradict described by Linville et al. (2004) claim to have achieved wherein DNA profiles in various liquid media including 70% ethanol, 95% ethanol and formaldehyde and dry form with up to six months of storage.

It should be emphasized that these authors have left their stored samples at 24ºC, with no change in storage temperature, as the samples described herein were held at ambient temperature, with an average of 28°C (± 2°C) in

order to simulate field conditions a criminal expert, especially in cases where the biological sample analysis laboratory is far from the site of collection.

When occurs the degradation of nuclear DNA, being directly affected by chemical and environmental factors and causing genetic information is lost in whole or in part, is sought alternative sources. Even with the use of markers such as short tandem repeats (STR), which are shorter and potentially recoverable, the degradation of the samples still affects obtaining genetic profiles.

An alternative is the use of mitochondrial DNA, which is more amenable to recovery in degraded samples, but being maternal lineage, with uniparental inheritance, can not be used for individualization of people (Wilson et al. 1995). Most articles that use of recovered samples from the digestive tract of insects, uses the hypervariable region of mitochondrial DNA to perform these analyzes (Lord et al., 1998, Wells et al., 2001, DiZinno et al., 2002, Linville and Wells, 2002, Li et al. 2011). We opted for the non-use of these markers, as we aimed to evaluate the techniques used for human identification and individualization in the forensic context.

Among the storage methods analyzed, which preserved the sample more effectively was the 4% formaldehyde. This formaldehyde concentration is commonly used to fix invertebrates, especially in young stages, and have the most soft body, such as the larvae of dipterae.

Even formaldehyde being demonstrated in the literature as interfering in DNA analysis, samples preserved in 4% formaldehyde generated full access to nuclear DNA to the markers used (Fig. 1).

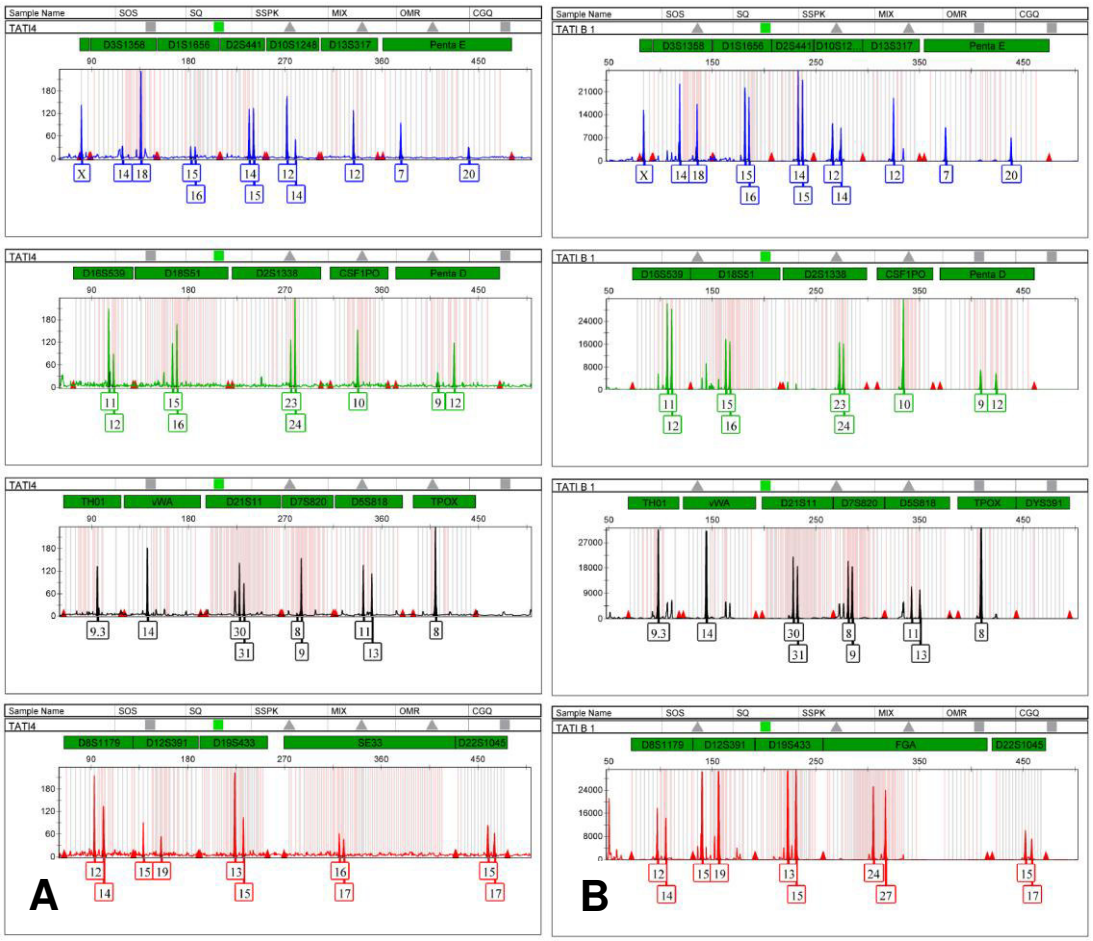

Figure 1: Electropherograms: A- human DNA sample recovered larvae muscoid flies obtained in samples stored in 4% formaldehyde; B- reference sample obtained from buccal swab.

The Patcan v.1.2 program, is used by forensic genetics laboratories to relate electropherograms in cases of traces of clashes. As the samples of human DNA obtained from intestinal tracts larvae muscoid flies, can be applied as an organic trace, the Patcan program was used to confirm, statistically the probabilities of the results. The probability of match for the samples questioned regarding the reference, was 100%.

The formaldehyde, aldehyde resulting from partial oxidation of alcohols (Ben-Ezra 1990), and used for fixation of invertebrate species, is the most widely used liquid medium for the preservation of tissues and corpses (Mies 1998). Studies have reported that formaldehyde is capable of degrading DNA in whole or in part when the sample is fixed in this reagent (Cheoker 2002).

In their study, Carvalho (2009) demonstrates the effectiveness of muscle samples from human cadavers analysis resulting in three concentrations of formalin, the concentration being at 5% did not harmful to analysis of nuclear DNA. However, usually the fixing of corpses and tissues is performed in

formalin concentration from 10%, which really affect the analysis of DNA, causing loss reading of some genetic loci (Greer 1991, Carvalho 2009).

Such evidence reinforces the possibility of fixing samples dipterae larvae in 4% formaldehyde, following the invertebrate storage protocols, without greater interference in DNA analysis. Even with lower peaks, it is still possible to obtain a complete human DNA profile.

There obtaining of intact DNA samples larvae fixed in 70% ethanol and 95% ethanol was possible. Although several studies in assert literature that alcohol is a good retainer for animals and apparently do not cause interference in the DNA analysis when the larval crop was determined in these media, there was degradation of samples with the passage of time (Linville, et al. 2004).

In the dry form, Linville et al. (2004) have reported that the samples without preservation media is degraded with time, it is not as effective in DNA analysis. A fact corroborated by the analysis of the sample in dry medium, which did not cause human DNA profile.

There were tested over longer periods of time fixing the fly larvae on the selected means, as to not obtaining satisfactorily DNA with 24 hours for most samples, it has not seen the need for further evaluation.

It can be concluded that the most efficient form of human DNA recovery from dipterae larvae is the dissection and analysis of direct food source so there is no loss of sample. If there is a need to fix the material for transport, the best approach appears to be the 4% formaldehyde that does not change the characteristics of the sample and has no significant degradation in the DNA contained in the digestive tract.

The scarcity of studies involving these storage processes samples with forensic purpose, undermine the use of human DNA recovery techniques in dipterae. It is expected that future studies, including over time and temperature variables may be performed to establish standard procedures to preserve the DNA samples.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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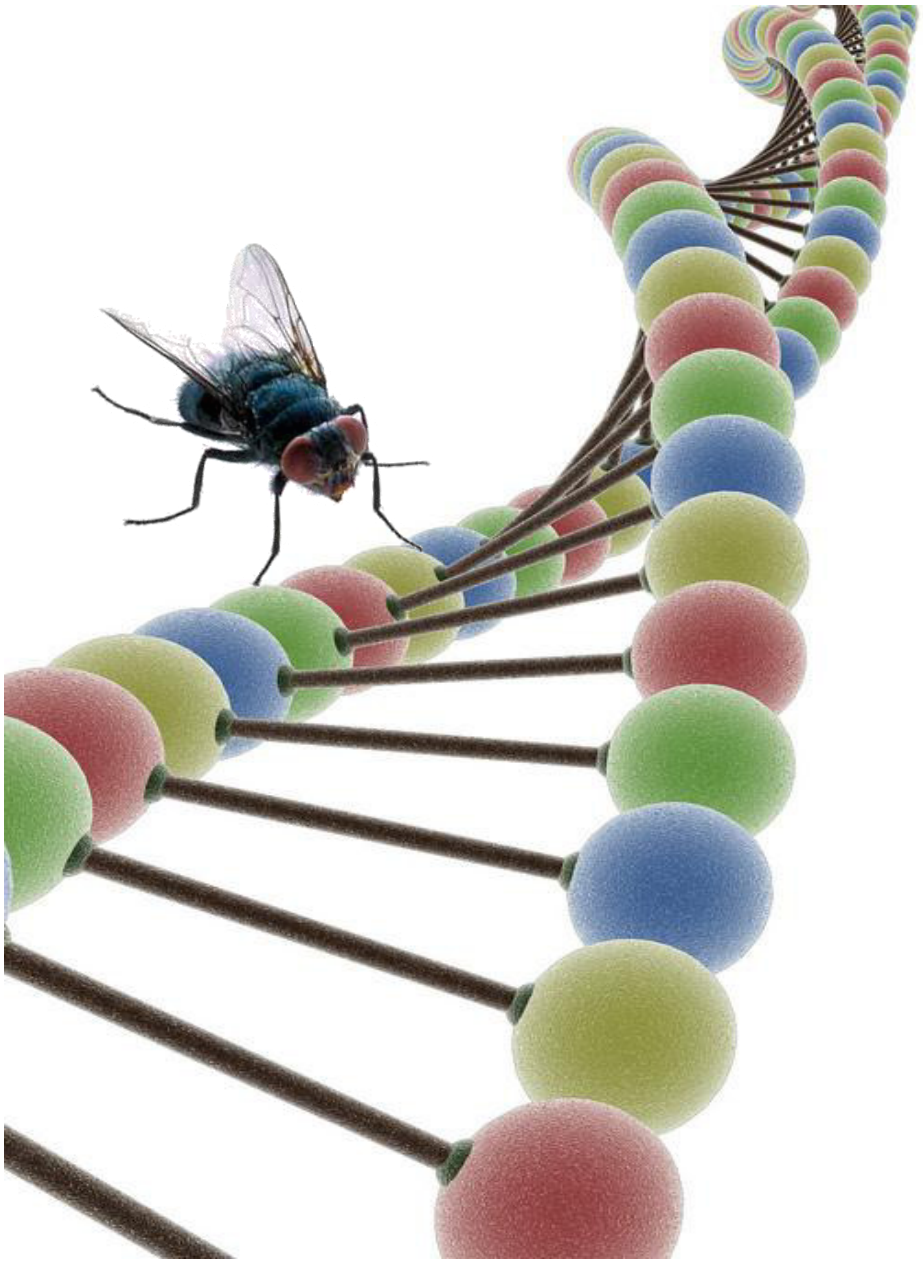
6. CONCLUSÕES



6. CONCLUSÕES

1. O melhor método para recuperação de perfis de DNA humano a partir de larvas de dípteros é a dissecção do papo larval;

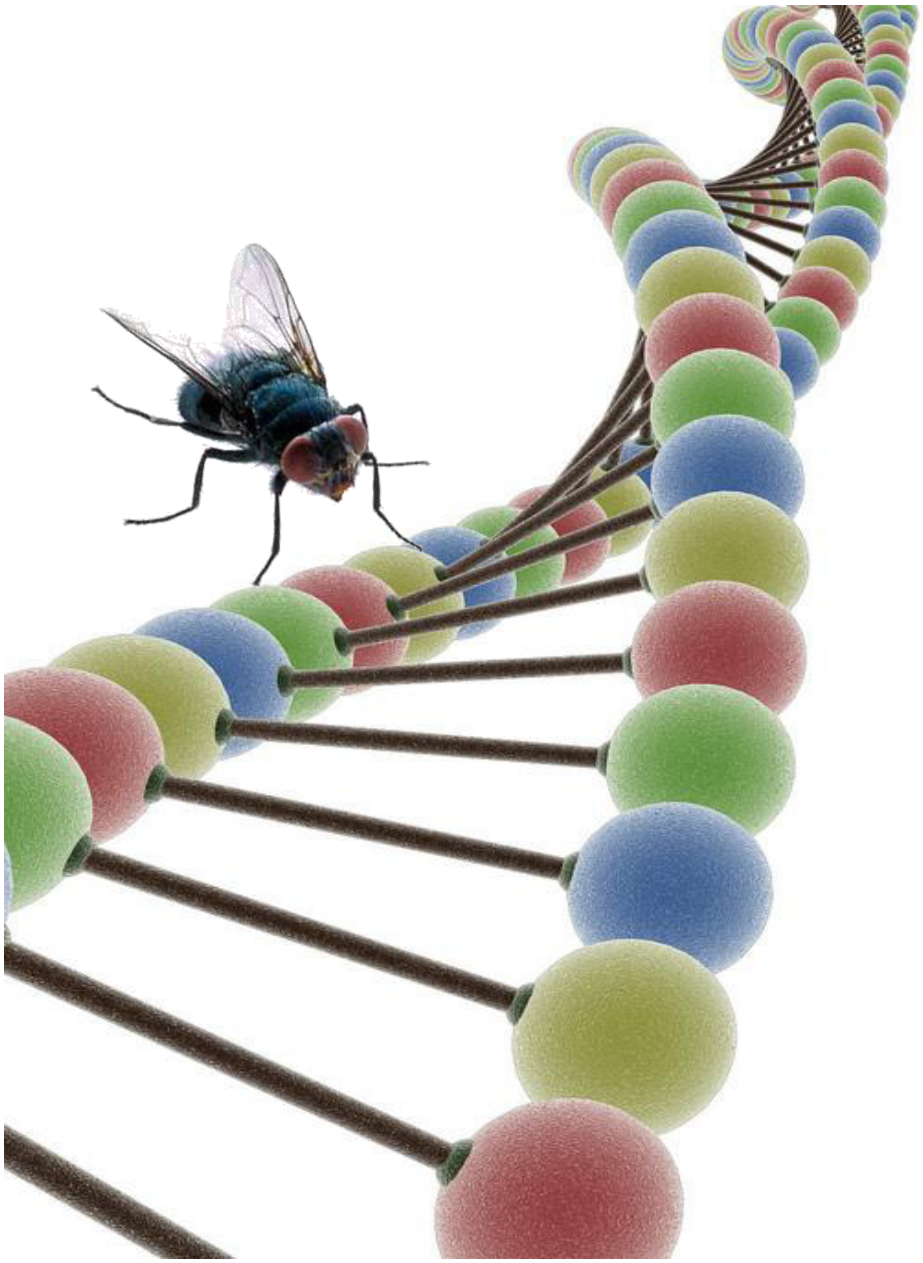
2. Deve-se realizar a extração de DNA das larvas tão logo estas percam contato com o alimento, caso contrário, a digestão prejudicará o procedimento de obtenção do perfil genético;

3. Os métodos de armazenagem convencionais para insetos, acabam por prejudicar o processo de análise de DNA humano no trato digestivo das larvas;

4. Dentre os meios de armazenagem testados, o formol 4% é o que preserva melhor a amostra externamente, bem como na análise de DNA;

5. Os kits comercias e protocolos comumente utilizados em genética forense são efetivos para genotipar o perfil de DNA humano obtido a partir de larvas de dípteros, bastando realizar pequenas modificações.

7. PERSPECTIVAS



7. PERSPECTIVAS

Com o crescente aumento da violência no Brasil e em especial na região Nordeste, há uma elevação no número de casos que necessitam, de uma intervenção pericial. Assim, faz-se necessário mais ferramentas que possam contribuir com a polícia na resolução das questões criminais.

As provas baseadas em análise científica hoje são as mais robustas que podem ser utilizadas profissionais da área forense. Uma das principais ferramentas, a análise do DNA, ganha destaque pela confiabilidade e segurança na produção da prova.

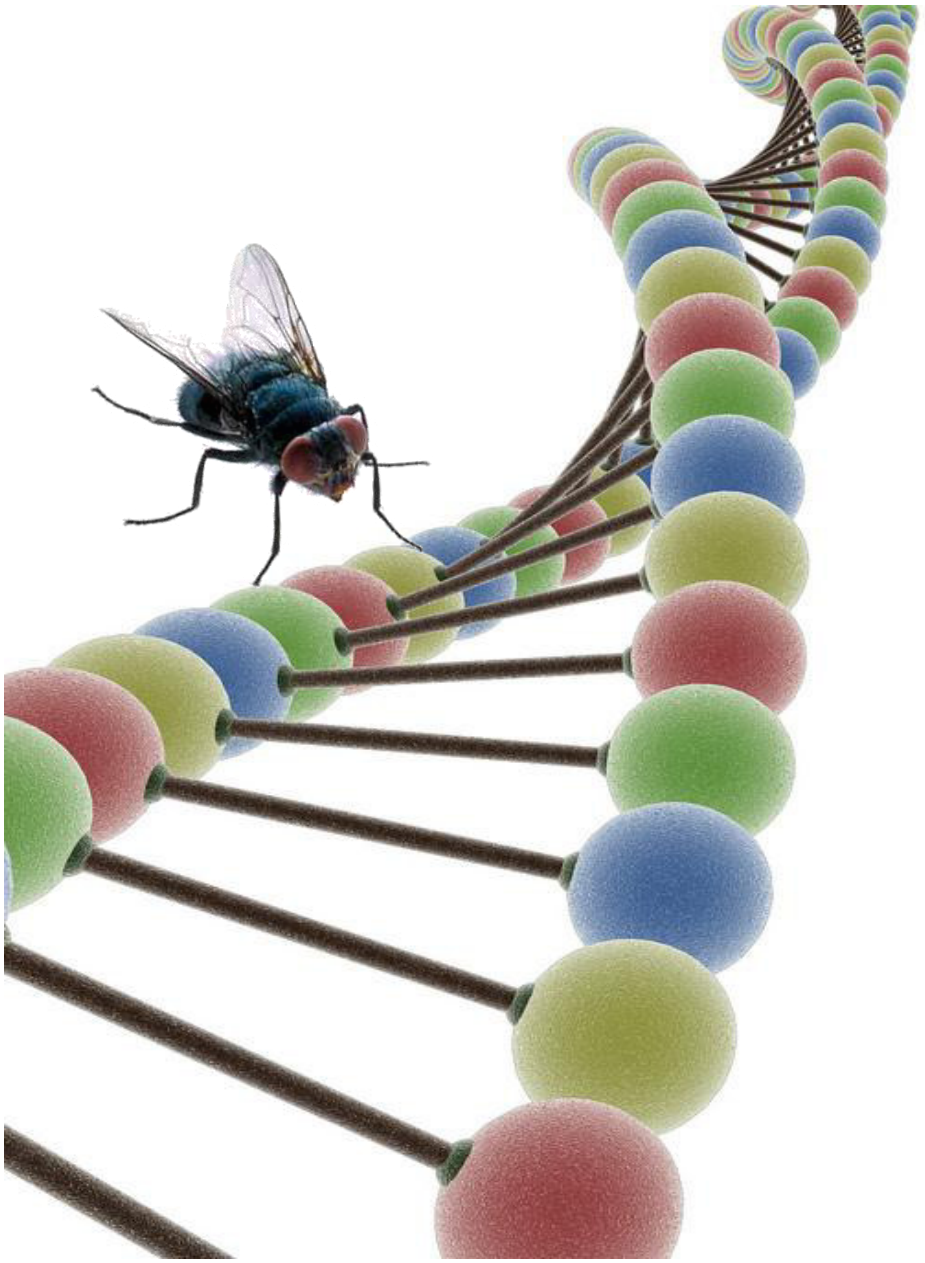
Diversas são as amostras que podem ser utilizadas para a análise do DNA e o conteúdo intestinal de larvas de dípteros muscóides são a mais recente alternativa. Diversos trabalhos já demonstram a eficácia da prova, mas ainda existe a necessidade de protocolos bem estabelecidos para validação e reprodução destas técnicas.

Como, em relação aos insetos, temos uma enorme variação quanto ao seu desenvolvimento, principalmente quando falamos em alterações de clima e temperatura, que influenciam diretamente nos seus hábitos de vida. Dessa forma, para cada localidade e cada grupo de indivíduos, deve-se haver um estudo prévio sobre seu comportamento e a influência na digestão e consequente degradação do DNA.

Espera-se que este trabalho possa ser um desencadeador de novos estudos visando o conhecimento específico e validação de protocolos que possam ser aplicados às espécies e condições locais. O estado de Pernambuco já possui diversos estudos sobre a fauna cadavérica local , que facilitam o conhecimento geral sobre as espécies.

É necessário agora que mais projetos, envolvendo o comportamento alimentar destes insetos e a sua interação com o DNA consumido do seu hospedeiro sejam realizados, aumentando o número de variáveis e validando os protocolos aqui preconizados. As parcerias entre as universidades e órgãos de perícia precisam ser fortalecidas para que esse tipo de pesquisa possa ser implementado com confiabilidade.

ANEXOS



Anexo 1: Protocolo para coleta, armazenagem e dissecção de larvas para obtenção de perfil de DNA humano

Coleta:

1. Coletar os imaturos com auxílio de pinças histológicas e armazenar em potes de 70mL cobertos com tecido fino (organza, por exemplo) para facilitar a oxigenação;

2. Coletar amostras dos imaturos para referência e armazenar em álcool 70% para posterior utilização como referencial taxonômico e/ou anatômico;

3. Dar preferência pela coleta de larvas maiores, possivelmente, as mais velhas e com maior tempo de alimentação;

4. Encaminhar as larvas o mais rápido possível para a dissecção, para que elas não percam o alimento armazenado no papo;

5. Iniciar o preparo para a dissecção.

Dissecção:

1. Limpar cada larva com água destilada e enxugar com papel toalha, para retirar as impurezas advindas do local de coleta;

2. Preferencialmente, com as larvas vivas, realizar a decapitação na região final do esqueleto cefalofaringeano, com auxílio de bisturi estéril;

3. Espremer a larva da parte terminal para a cefálica, retirando todo o sistema digetório;

4. Localizar a região do papo larval e com auxílio de bisturi, separá-lo do trato digestório;

5. Imediatamente, proceder com os protocolos de extração de DNA;

6. Após a extração do DNA, o mesmo poderá ser estocado em freezer -20ºC.

Obs.: Caso haja a necessidade de armazenagem antes da extração, o meio utilizado, para melhor preservação do inseto é o formol 4%.

Anexo 2: Protocolo de extração de DNA utilizando o Kit DNA IQ™ (Promega).

1. Preparo de soluções:

   1-a) Preparar Lysis Buffer + DTT 1M, adicionando 1µL de DTT para cada 100µL de Lysis Buffer. Calcular para cada amostra 300µL;

   1-b) Preparar Wash Buffer + álcoois, utilizando uma parte de etanol, uma de isopropanol e duas de Wash Buffer 2x. Calcular 320µL para cada amostra;

2. Dividir o Swab ou recortar o suporte contendo as manchas em pequenos fragmentos com auxílio de bisturi; Alojar a amostra em um tubo de 1,5 ou 2mL, dependendo do tamanho e quantidade;
3. Adicionar Lysis Buffer + DTT em quantidade suficiente para mergulhar completamente a amostra, usualmente 200µL;
4. Homogeneizar com o vórtex, incubar a 70ºC por 30 minutos;
5. Separar todo o líquido e transferir para um tubo novo, através de centrifugação em "spin baskets" ou aspirando a parte líquida; cuidado para não transferir pequenos fragmentos ou fiapos, que podem interferir com as partículas magnéticas;
6. Adicionar 7µL* de partículas magnéticas. Certificar-se de manter as partículas em suspensão antes de pipetar, recomenda-se vortexar o frasco por 10 segundos antes de aspirar a suspensão. *Amostras referência – adicionar 3,5µL;
7. Vortexar a amostra com as partículas por 5"; incubar a temperatura ambiente (25ºC) por 10 minutos;
8. Vortexar novamente e imediatamente colocar o tubo na estante magnética para imobilizar as partículas; aspirar todo o líquido e descartá-lo;
9. Adicionar 100µL de Lysis Buffer + DTT e repetir o passo 8;
10. Adicionar 100µL de Wash Buffer + álcoois, vortexar novamente, imobilizar as partículas no magneto; aspirar todo o líquido e descartá-lo;
11. Repetir o passo 10 mais duas vezes, totalizando três lavagens com Wash Buffer + Álcoois; certificar-se de remover todo o líquido ao final da

última lavagem;

12. Deixar o tubo aberto em temperatura ambiente para evaporação dos álcoois por 5 minutos. Obs.: Não deixar secar por mais de 20 minutos;
13. Adicionar 50µL de Tampão de Eluição do Kit; vortexar por dois segundos;
14. Incubar a 65ºC por 5 minutos; retirar o tubo do banho, vortexar novamente, e, sem deixar o material esfriar, imobilizar as partículas no magneto, retirar a parte líquida (contendo o DNA) e transferi-la para um novo tubo;
15. DNA purificado.

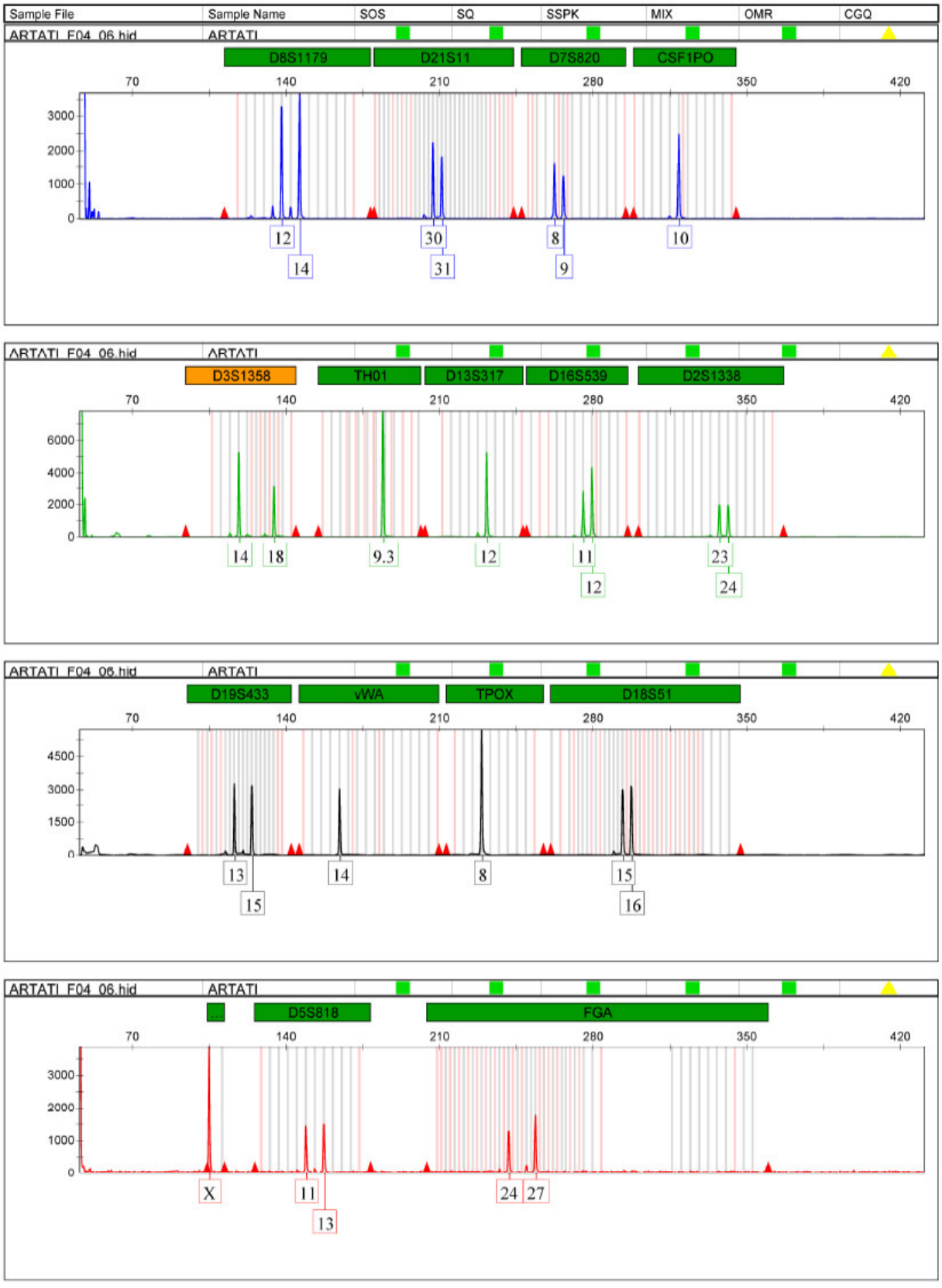
Anexo 3: Protocolo de extração de DNA pelo método orgânico.

1. A amostra deve ser suspensa em 300µL de tampão SEB, 7,5µL de pk (20mg/mL) e 3, 75µL DTT (30mg/5mL de SEB);
2. Todas as amostras devem ser vortexadas por 5 segundos e centrifugadas a 13400rpm por 2 minutos;
3. Incubação a 56°C por 2 horas;
4. As amostras incubadas são tratadas com solução de fenol:clorofórmio:álcool isoamílico (25:24:1) em quantidade 1:1 com o volume de SEB;
5. As amostras tratadas são novamente centrifugadas em alta velocidade por 2 min;
6. As fases aquosas são transferidas para Microcon™ de 100 contendo 100µL de tampão Tris-EDTA (10mM de Tris, 0,1mM de EDTA, pH 8,0);
7. Os tubos Microcon™ são centrifugados a 5000 rpm por 5min. O sobrenadante é descartado e 100µL de TE é adicionado novamente aos tubos Microcon™;
8. As amostras vão novamente em velocidade máxima por 20min. Após a centrifugação os tubos são descartados e 200µL de TE estéril é adicionado a parte superior do Microcon™;
9. Tubos de recuperação da amostra foram adicionados a um aparelho de filtração;
10. As amostras são agitadas em vórtex brevemente em velocidade média com o tubo de recuperação voltado para cima;
11. Os tubos de recuperação e o aparelho de filtração são invertidos e centrifugados a 12.000g por 5min;
12. O volume recuperado é de aproximadamente 200µL.

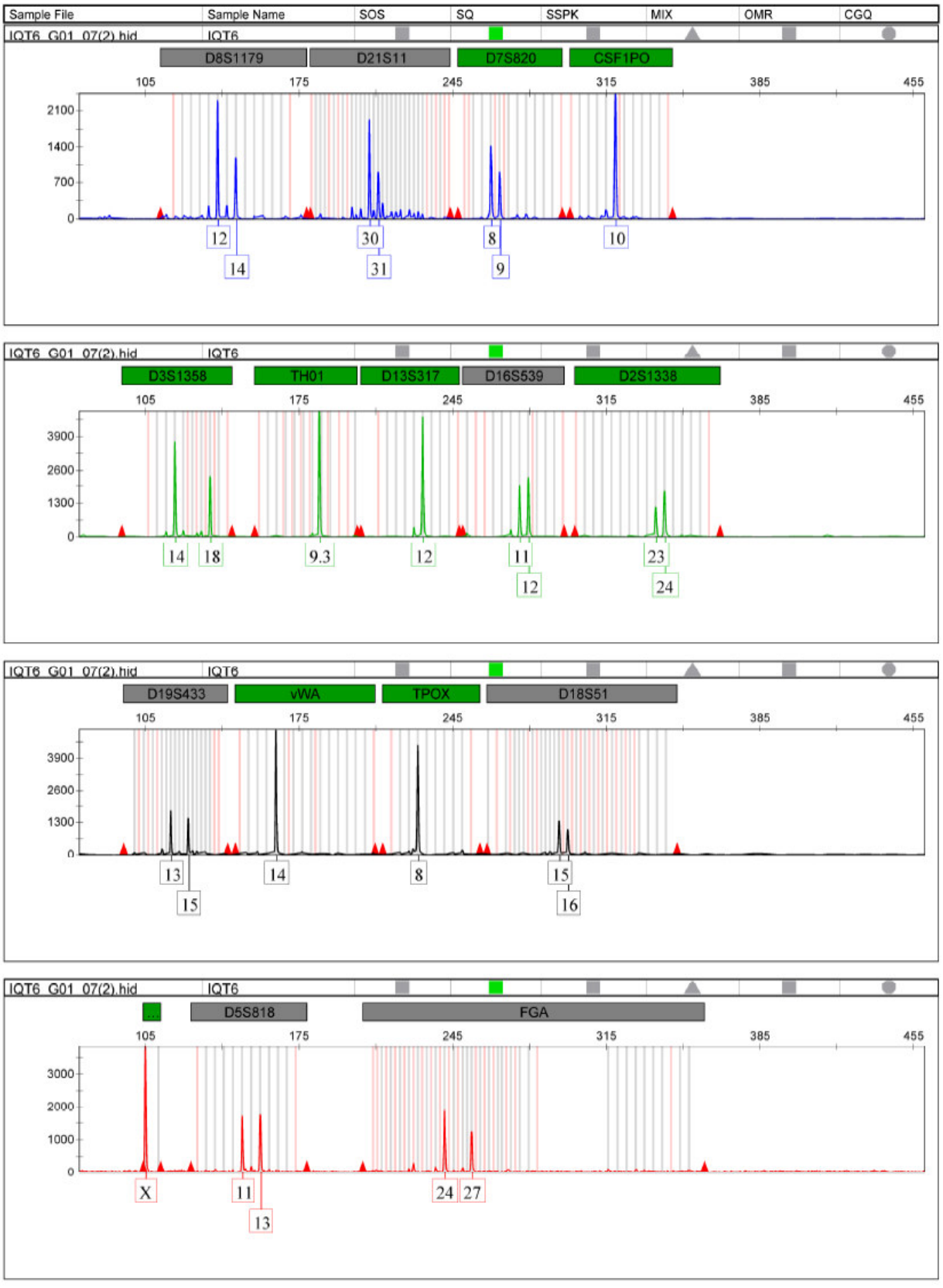
Tampão SEB

- Tris 1.0M, pH 8,0 – 10mL
- EDTA 500nM, pH 8,0 – 20 mL
- NaCl – 5, 84g
- SDS 20% – 100 mL
- Água Mili-Q (ou equivalente) – completar 1L.

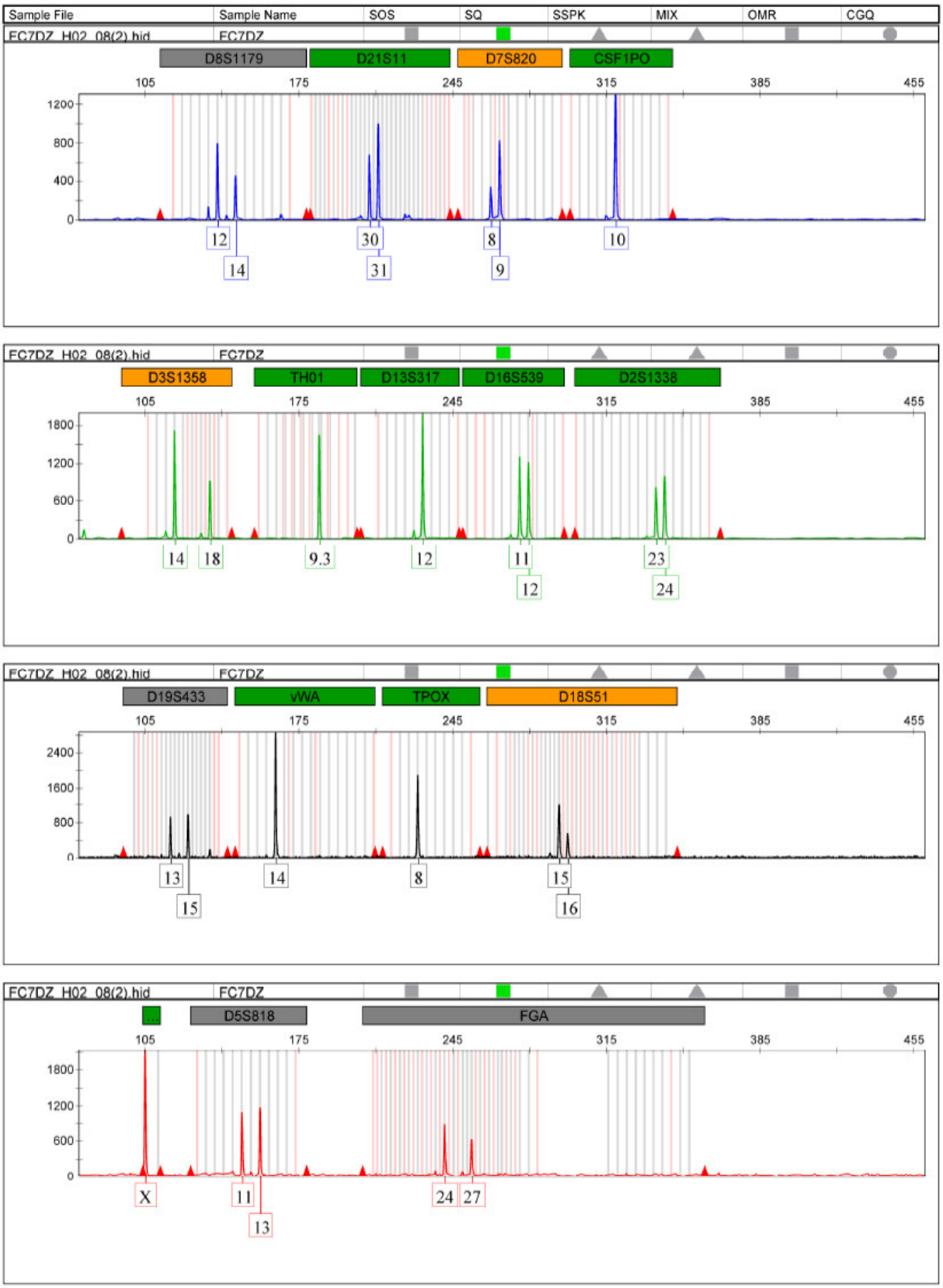
Anexo 4: Eletroferograma da amostra referência extraído com Kit DNA IQ™ (Promega) e amplificado com AmpFℓSTR® Identifiler® Plus PCR kit (Applied Biosystems) para 15 marcadores e amelogenina.



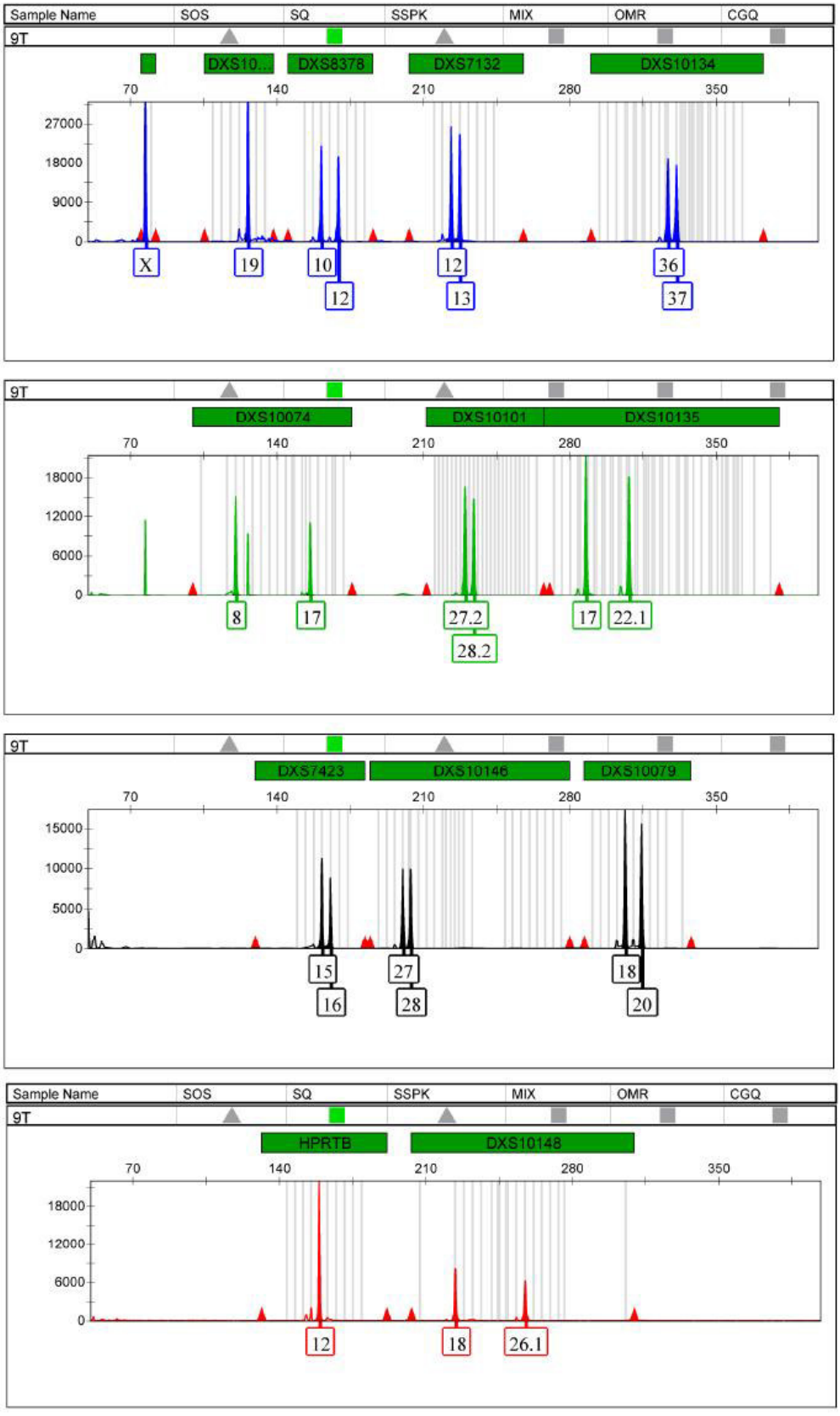
Anexo 5: Eletroferograma da amostra larval extraída com Kit DNA IQ™ e amplificado com AmpFℓSTR® Identifiler® Plus PCR kit (Applied Biosystems) para 15 marcadores e amelogenina.



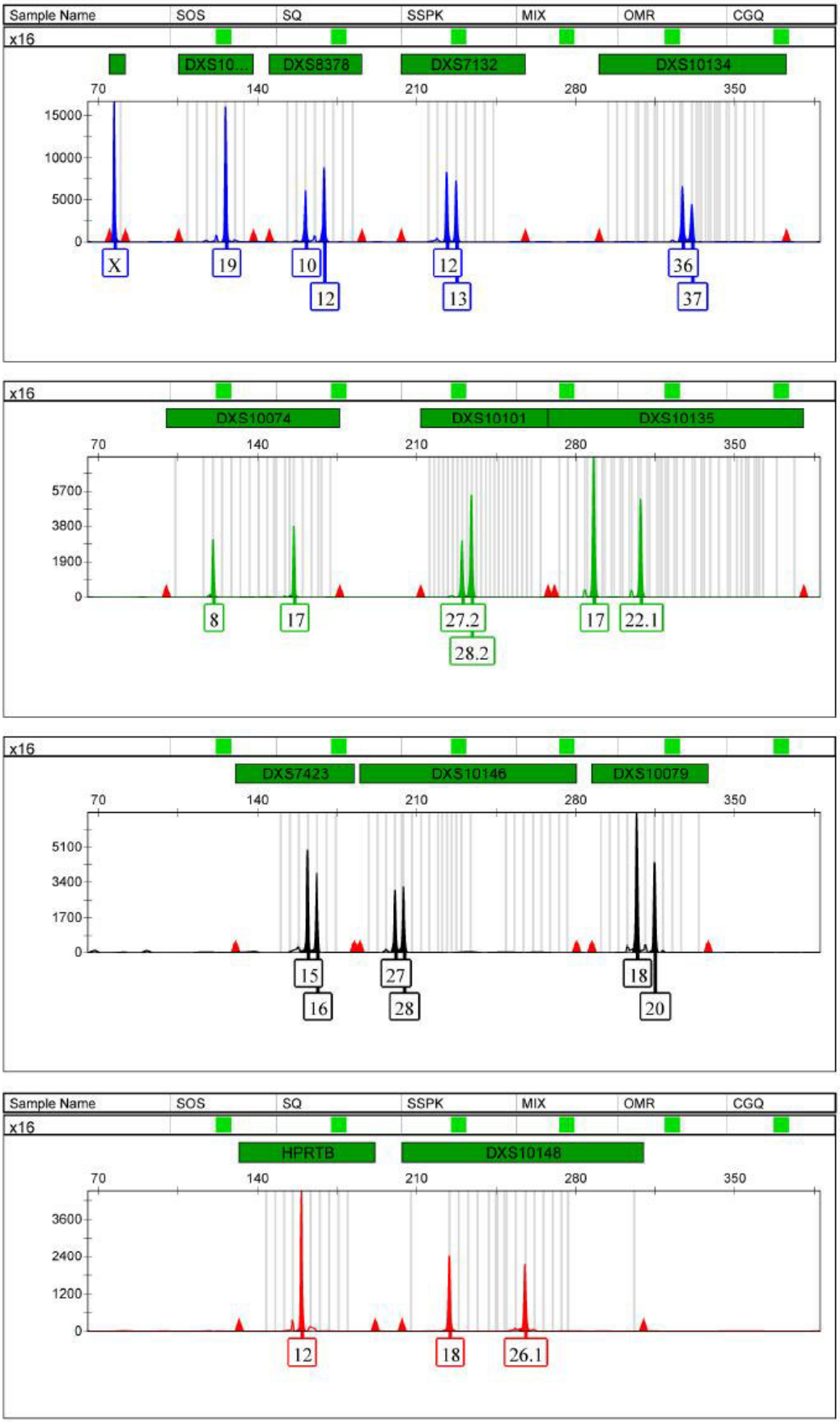
Anexo 6: Eletroferograma da amostra larval extraída com Fenol-Clorofórmio e amplificado com AmpFℓSTR® Identifiler® Plus PCR kit (Applied biosystems) para 15 marcadores e amelogenina.



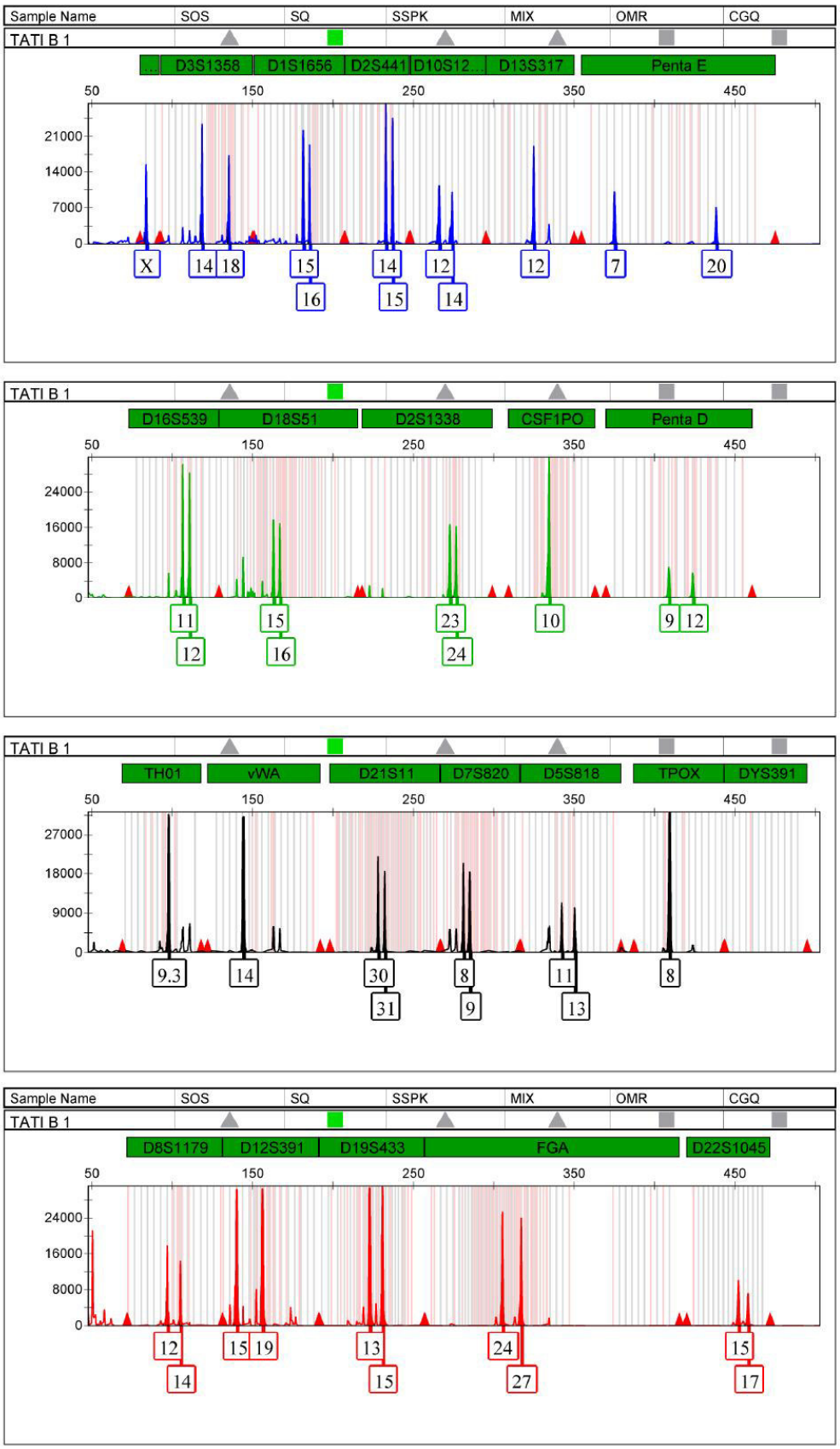
Anexo 7: Eletroferograma da amostra referência extraída com Fenol-Clorofórmio e amplificado com kit Argus-X (Qiagen) para 12 marcadores e amelogenina.



Anexo 8: Eletroferograma da amostra larval extraída com Fenol-Clorofórmio e amplificado com kit Argus-X (Qiagen) para 12 marcadores e amelogenina.



Anexo 9: Eletroferograma da amostra referência extraída com Fenol-Clorofórmio e amplificada com o kit PowerPlex® Fusion System 6C (Promega) para 24 marcadores e amelogenina.



Anexo 10: Eletroferograma da amostra larval extraída com Fenol-Clorofórmio e amplificada com o kit PowerPlex® Fusion System (Promega) para 22 marcadores e amelogenina.