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microRNAs em abelhas: busca e caracterização de proteínas da via, miRNAs e seus alvos

PATOS DE MINAS - MG

DEZEMBRO DE 2022

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Biotecnologia como requisito para a obtenção do título de Mestre em Biotecnologia.

Orientador: Prof. Dr. Matheus de Souza Gomes

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Dissertação apresentada com o objetivo de obtenção de título de Mestre em Biotecnologia, a qual dedico e agradeço, primeiramente, **A Mim Mesmo**.

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"Chega, o momento em que é preciso se abdicar, pra caminhar." **Djonga, Santa Ceia**

"Tentando dar meu melhor na minha pior fase." **Djonga, JUNHO DE 94**

"E o mundo tem sido pequeno demais, pra nós/ E a vida tem dado conquista demais, pra nós" **Djonga, ETERNO**

"Fala aí se eu não sou um cara forte, ultrapassei essas barreira ileso." **Djonga, LADRÃO**

"Você só vai ser o melhor do Brasil, depois que for o melhor da sua rua." **Djonga, O Cara de Óculos**

"É melhor desistir ou viver humilhado? Coisas que passam na mente de gente que vem de onde vem..." **Djonga, Xapralá**

"E foi assim, que eu me vi / Tu é igual nós / Dói igual em todo mundo." **Djonga, a cor púrpura**

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"EU SOU A CONTINUAÇÃO DE UM SONHO"

BK

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RESUMO

As abelhas são insetos milenares, tem seu surgimento muito antes do homem, há cerca de 100 milhões de anos. Tão antiga também é sua domesticação, sendo cultuadas como símbolo de riqueza e perseverança no antigo Egito. Pertencentes ao filo dos Artrópodes, classe Inseto e ordem dos Hymnoptera, acredita-se que as abelhas se originaram de grupos de vespas que, ao longo de milhões de anos de evolução, tiveram alterações quanto a dieta, deixando de se alimentar de insetos e ácaros, e passaram obter nutrientes através do néctar e pólen das flores. Há cerca de 20 mil espécies no mundo, no Brasil 1678 descritas, mas estudiosos dizem que esse número é próximo de 2500, colocando o Brasil como uma das maiores diversidades do planeta. As abelhas são animais de hábitos e comportamentos incrivelmente complexos, principalmente no que tange às características de Eusociabilidade, sendo classificadas em sociais e solitárias. O estilo de vida das abelhas é um dos motivos pelos quais esses animais vem sendo objeto de inúmeras pesquisas que buscam elucidar fatores que influenciam nesse ponto. Duas moléculas vem recebendo uma atenção especial em pesquisas desse campo, são a vitelogenina e o hormônio juvenil, que já demonstraram ser de grande importância para o ciclo de vida das abelhas e influenciam na sua diferenciação quando ainda da postura de ovos e alimentação dos mesmos nas células de cria dos ninhos. O hormônio juvenil é responsável pelo desenvolvimento embrionário desses insetos, estando presente em processos de maturação larval, purpal e metamorfose, e ainda em diversos outros processos biológicos que influenciam na reprodução e comportamento. Já a vitelogenina tem ação direta na diferenciação das larvas quanto ao sistema de castas que impera na colmeia. Trata-se de uma proteína essencial na reprodução, já que é o principal alimento para o embrião, além de sua disponibilidade também diferenciar o desenvolvimento em rainha ou operária. O presente trabalho visa a busca e caracterização de uma terceira classe de moléculas que possam interagir na função biológica do vitelogenina e do Hormônio Juvenil. MicroRNAs são pequenos RNAs não codificadores, que atuam no controle e regulação da expressão gênica a nível pós-transcricional. Este estudo busca utilizar ferramentas de bioinformática para a busca e caracterização de proteínas que compõe a via de miRNAs, além de miRNAs e seus alvos, no genoma de oito diferentes espécies de abelhas, tanto sociais quanto solitárias, focando em sua ação na modulação de Vitelogenina e Hormônio Juvenil.

Palavras-chave: Abelhas, microRNAs, Vitelogenina

Abstract

Bees are millennial insects that had their emergence long before humans, something like hundreds of millions of years ago. Their domestication is as old as being worshiped as a symbol of wealth work and perseverance in ancient Egypt. Belonging to the phylum Arthropoda, class Insecta, and order Hymenoptera, it is believed that bees are originally from a group of wasps that, for millions of years of evolution, had dietary modifications, leftover a diet based on insects and mites, and starting to obtain nutrients by nectar and pollen from flowers. There are about 20 thousand species around the world, with about 1678 described in Brazil, but it is expected that the real number is about 2500 species, attributing to Brazil as one of the biggest diversities on the planet. Bees have an amazing and complex habits and behaviors, mainly about de Eusociability, lifestyle, where they can be characterized as social bees, solitary bees and parasite. The bee's lifestyle is one of the most characteristics that has made this animal of the subject of many researchers trying to elucidate the factors of influence in this aspect. Two molecules have been the central point in this study, Vitellogenin and Juvenile Hormone, that is because these molecules already showed essential importance in the life cycle of bees and because they influence bees' differentiation, even during the layoff of eggs. Juvenile hormone is responsible for the embryonic development in this insect, been present in the maturation of larval, pulpal stages, and metamorphosis, and yet in a variety of others biological processes that impact the reproduction and behavior in bees. Vitellogenin play a role in larval differentiation through the caste system used in hive organization. It is an essential protein involved in reproduction, being the central nourishment to embryo, it is noticed that their availability is capable of differentiating the egg into a queen or worker. This study aims to search and characterizes a third molecule able to interact in the biological function with both of them. microRNAs are small non-coding RNAs, and they control and regulatecontrol and regulate gene expression in post-transcriptional ways. This analysis will use bioinformatic tools to search and characterize proteins involved in the miRNA pathway and miRNAs and their targets in the genome of eight different species of bees, as both social and solitary ones, focusing on their action in Vitellogenin and Juvenile Hormone modulation.

Keywords: Bees, microRNAs, Vitelogenin

Sumário

1 INTRODUÇÃO

1.1 Problema de pesquisa

Compreendendo mais de 20 mil espécies ao redor do mundo, as abelhas estão incluídas na Ordem Hymenoptera e na Superfamília Apoidea, subdivididas ainda em aproximadamente 4 mil gêneros. Dentre esses gêneros, as espécies são comumente conhecidas por uma característica fenotípica de fácil observação, a presença ou não de ferrão. Nos criadouros há utilização de ambas com e sem ferrão. Como exemplo de espécie de ferrão temos a Apis melífera, devido à sua produção exacerbada de mel e fácil adaptação, seu manejo é preterido entre os produtores (Milfont et al., 2009; Neves & Viana, 2002). Quanto às espécies sem ferrão, temos as pertencentes à tribo Meliponini, que tem uma ampla distribuição e imensa biodiversidade no Brasil, representando até 40% das espécies mais observadas nas flores, também tem seu manejo facilitado por se tratarem de indivíduos eussociais (Barros et al., 2022; Neves & Viana, 2002).

Ainda sobre a classificação em abelhas, além de características físicas, o comportamento das mesmas as diferencia e dá razão à diversos estudos sobre as espécies. O conceito de eussociabilidade dentro de espécies animais diz respeito principalmente a complexos tipos de comportamento, como tomada de decisão dentro da comunidade; isso inclui principalmente a organização da colmeia na ajuda mútua para o cuidado com os indivíduos mais novos, a organização em grupos, seleção de indivíduos para reprodução (no caso das abelhas, distinção entre rainha e operárias), e ainda sobreposição entre gerações (Plowes, 2010).

1.2 Hipóteses

1.2.1 A via de biossíntese de microRNAs é conservada entre as oito espécies de abelhas.
1.2.2 Há conservação de importantes microRNAs envolvidos em diferentes processos biológicos nas oito espécies de abelhas.
1.2.3 Há microRNAs que tem como potencial alvo a Vitelogenina.

1.3 Objetivos

Objetivo geral: Este trabalho propõe a análise de genoma para busca de microRNAs em oito diferentes espécies de abelhas, proteínas que compõem a via, e seus potenciais alvos.

1.4 Objetivos específicos:

Os objetivos que serão descritos à seguir serão aplicados às oito espécies de abelhas à serem trabalhadas neste projeto.

• Utilizar bancos de dados públicos para obtenção dos dados de genoma e transcritoma.

• Identificar as prováveis proteínas que compõem a via de microRNAs.

• Caracterizar as prováveis proteínas Argonauta, Dicer e Drosha quanto aos seus resíduos de aminoácidos de sítio ativo, domínios conservados e análise filogenética.

• Identificar e caracterizar precursores de microRNAs.

• Identificar microRNAs maduros.

• Recuperar microRNAs específicos de interesse e caracterizá-los quanto à conservação por meio de alinhamento global, estrutura secundária e análise filogenética.

• Identificar ospotenciais alvos dos microRNAs.

1.5 Justificativa

O Brasil é signatário da Convenção da Diversidade Biológica, que fez parte da Eco-92, onde espécies consideradas polinizadoras são reconhecidas como fundamentais na manutenção da biodiversidade mundial (Barbiéri & Francoy, 2020). Há anos a comunidade de abelhas vem enfrentando consecutivos eventos de diminuição drástica de suas populações, tanto em criadouros quanto em indivíduos selvagens. Há diversos motivos para tais eventos, como mudanças climáticas, e a degradação de seu habitat natural. Esse último é o mais preocupante, uma vez que os indivíduos perdem a flora silvestre, eles são obrigados a procurar alimento em plantações que tomaram o ambiente natural, e que geralmente estão carregadas de inseticidas (*Colapso de Colônias de Abelhas Africanizadas*, 2009; Jaffé et al., 2019). Este cenário demonstra a necessidade de incentivo à pesquisa envolvendo Abelhas, quão maior for o conhecimento a respeito da biologia destes insetos, melhorias podem ser feitas no manejo e proteção, tanto de colmeias selvagens quanto as domesticadas.

2 REFERENCIAL TEÓRICO

2.1 Generalidades – Polinização e mortandade

A vida das abelhas é como um poço mágico, quanto mais se tira, mais há para tirar – Karl von Frisch (1886 – 1982). Essa frase foi dita pelo pesquisador etologista citado, que recebeu postumamente, em 1973, o prêmio Nobel de fisiologia pelo primeiro estudo que visava a elucidação de padrões inatos de comportamento em animais. O trabalho de von Frisch descrevia como as abelhas encontravam néctar nas flores e logo depois voavam em um padrão específico para mostrar às outras abelhas onde foi encontrado o néctar coletado (Plowes, 2010).

Estudiosos avaliam a coevolução entre plantas e abelhas principalmente pela mutualidade da relação, enquanto as plantas fornecem o alimento na forma do néctar, as abelhas contribuem com a polinização dessas plantas. O pólen é o gameta masculino da flor, ao pousar, o inseto usa os "pelos" que recobrem todo seu tronco para reter as partículas de pólen. Essas partículas são consequentemente carregadas para outras flores pelos mesmos animais e acabam sendo depositados na estrutura reprodutiva feminina (estigma) da mesma flor ou de outras flores da mesma espécie, esse é o evento chamado polinização. Esse acontecimento não é uma exclusividade das abelhas, mas elas são as que o fazem com maior efetividade. Isso se dá, principalmente, porque elas se alimentam de pólen ao longo de toda vida, fazendo com que a busca pelo alimento seja constante; enquanto outros insetos como as vespas, só se alimentam dessas estruturas já na fase de vida adulta (Rhoades, 2013).

No Brasil, cerca de 60% das plantas cultivadas com interesse comercial, seja alimentação, produção animal, dentre outros, tem a polinização animal como fator indispensável (Giannini et al., 2015). Apesar de ser uma prática pouco utilizada em plantações de larga escala, são diversas as contribuições da polinização por abelhas. Em um estudo conduzido por Björn Klatt (2014) e colaboradores, procurou-se avaliar a diferença nos frutos de morango após serem expostos a polinização natural por abelhas livres, contra a auto-polinização e a polinização por ação do vento apenas (Klatt et al., 2013) . Os resultados foram surpreendentes, mostrando que a polinização por abelhas proporciona ao fruto um maior tempo de prateleira e melhor qualidade pós colheita, o que impacta diretamente no seu valor comercial.

Devido a contribuição comercial e do impacto financeiro que as abelhas apresentam para a economia, a mortandade desses insetos é um evento que tem chamado atenção nos últimos anos. As abelhas estão suscetíveis aos mesmos vários fatores que comumente afetam as populações animais como mudanças climáticas, perda de habitat, disponibilidade de alimento, dentre outros. Este último tem uma peculiaridade, as abelhas

também são conhecidas pela sua predileção, ou não, pelo pólen de plantas específicas (Murray et al., 2009). Robertson, em 1925, foi quem primeiro descreveu que a busca por pólen não acontece aleatoriamente para as abelhas, e as dividiu entre Monoléticas, que coletam pólen de uma espécie específica, Oligoléticas, que coletam de duas espécies ou mais porém de um mesmo gênero, e Poliléticas, que coletam pólen de mais que quatro gêneros de até duas famílias diferentes (Society, 2013). Estes conceitos foram trabalhados e atualizados recentemente por outros autores, que propuseram outra divisão, por exemplo uma em que as Mesoléticas coletam pólen de mais que quatro gêneros de plantas pertencentes a até três famílias diferentes, enquanto que as Poliléticas coletariam em plantas de inúmeros gêneros e pelo menos quatro famílias diferentes (Müller & Kuhlmann, 2008). Essa característica deixa esses animais ainda mais susceptíveis ao desaparecimento quando de eventos catastróficos que eliminem espécies de plantas endêmicas, deixando as espécies de abelhas monoléticas sem alimento disponível.

Além das causas já conhecidas que incidem sobre todos os animais de vida selvagem, a partir do inverno de 2006, EUA e Europa anotaram perdas significativas em colônias de criadores, chegando a 30% de colmeias de *Apis mellifera* (Pires et al., 2016), e até então, sem uma causa provável. Diante dessas perdas, diversos grupos se formaram no intuito de investigar as causas de mortandade nessas colônias, não se chegou a uma causa específica, mas sim em um diagnóstico de que uma gama de eventos correlacionados (patógenos, mudanças climáticas, parasitas, uso de agrotóxicos, dentre outros) estavam causando o problema; esse fenômeno passou a ser tratado como síndrome, sendo nomeado como CCD, "Colony Collapse Disorder" (vanEngelsdorp et al., 2009).

Fato é, o conhecimento da biologia básica desses insetos é de suma importância para delinear ações de conservação eficientes. O conhecimento de fatores que controlam a população e as comunidades, e como as abelhas reagem por si próprias à esses fatores, permitem um manejo focado tanto em espécies específicas, como para gêneros inteiros (Murray et al., 2009).

2.2 Níveis de Socialização

Conceitualmente, a sociabilidade dentro de qualquer grupo é dita como o propósito que leva os indivíduos a interagirem entre sim, sendo uma característica fundamental para determinados organismos (Ward & Webster, 2016). Em abelhas, essa

característica é marcada por fatores como sobreposição de gerações, cuidados com os membros da comunidade, divisão de trabalho dentro da colmeia, reconhecimento de indivíduos da mesma colmeia e de intrusos, e exclusividade de um único indivíduo responsável pela reprodução (Danforth, 2002; Hewlett et al., 2018). A evolução das espécies traça um caminho compreendendo desde as espécies solitárias, primitivamente eusociais e altamente eusociais, e é considerada uma das maiores transições evolutivas da terra, sendo um evento que fascina biólogos e pesquisadores da área (Szathmáry & Smith, 1995; Woodard et al., 2011).

Um ponto chave que distingue os animais eusociais dos que se desenvolvem solitários é o altruísmo com relação aos demais integrantes da colmeia, onde os indivíduos mais velhos são responsáveis pelo cuidado aos mais novos (Michener, 1969). Esse comportamento influencia em uma outra característica das abelhas eusociais, que é o próprio desenvolvimento da sociabilidade e o cuidado com o ninho. As abelhas sociais, como por exemplo a *A. mellifera*, tem que desenvolver alguma ferramenta que as permitam identificar os indivíduos da colmeia, pois apenas os moradores da comunidade têm passe livre, o que ocorre normalmente a partir do olfato. Isso possibilita aos insetos que ficam na entrada do ninho, identificar e permitir, ou não, a entrada dos demais (Breed & Stiller, 1992; van Zweden & d'Ettorre, 2010). Essa capacidade de identificar os moradores da colmeia é desenvolvida nas primeiras 24 horas após a eclosão dos ovos, e é dependente da exposição das abelhas ao meio, e ao tempo que as mesmas gastam socializando umas com as outras (Breed & Stiller, 1992; D'Ettorre et al., 2006; Downs & Ratnieks, 1999; van Zweden & d'Ettorre, 2010). O tempo de exposição entre os insetos também dita o nível de sociabilidade dos mesmos, onde mesmo espécies já descritas como altamente sociais perdem essa capacidade de sociabilidade quando em isolamento, assim como a capacidade de predileção pelas abelhas da mesma colmeia (Hewlett et al., 2018).

A característica de sociabilidade também tem um impacto considerável na questão populacional de cada espécie, afetando profundamente sua capacidade de autoconservação. Este fato está diretamente ligado a organização dos indivíduos capazes de reproduzir dentro da comunidade. Assim, temos uma relação em que quanto mais social a espécie, maior a tendência de um menor número de indivíduos para um censo geral, uma vez que organizacionalmente as espécies sociais tem apenas um indivíduo encarregado de se reproduzir, enquanto que nas espécies solitárias cada qual é capaz de colaborar com a perpetuação da espécie através da reprodução (Murray et al., 2009).

Apesar de toda a ideia construída em torno das grandes colmeias de abelhas, de todos os estudos sobre como as comunidades se organizam, de como esses insetos desenvolvem mecanismos tão precisos para viver em sociedade, o outro lado, das espécies solitárias, também abriga comportamentos tão formidáveis quanto.

Com cerca de 78% das espécies de abelhas já catalogadas classificadas como solitárias, essas espécies são de certa forma negligenciadas pelos especialistas, com uma literatura longe de ser tão vasta quanto o que há descrito para as espécies sociais. As espécies solitárias se distinguem das espécies sociais em praticamente todos os seus hábitos, cada ninho é composto por um indivíduo fêmea que é rainha e operária, realizando todas as obrigações da colmeia. Ela quem constrói e protege o ninho, reproduz, busca alimento, dentre outras tarefas (Morato & Martins, 2006). Neste caso, há ninhos menores, apenas para abrigar a abelha responsável e sua ninhada, normalmente construídos em buracos no solo, na madeira, ou no caule de plantas (Linsley, 1958).

No que tange à capacidade de polinização, as preferências de abelhas solitárias também se diferem das sociais. Espécies sociais como *A. mellifera* e *Bombus spp* tem a capacidade de buscar alimento à quilômetros de distância do ninho, e estas têm predileção por grandes floradas; enquanto que espécies solitárias como *Lasioglossum spp* se deslocam por apenas algumas dezenas de metros para a coleta de pólen, e podem coletar tanto de flores esparsas como de grandes plantações; bem como ter preferência por determinadas espécies de plantas (Bänsch et al., 2021; Linsley, 1958). Isso torna as abelhas sociais mais cotadas para o trabalho de polinização, pelo número de indivíduos, e sua capacidade de cobrir áreas maiores (Leonhardt et al., 2011).

2.3 Vitelogenina (Vg) e Hormônio Juvenil (HJ)

Como já descrito acima, as abelhas são insetos com inúmeras particularidades em seu comportamento, onde a maioria ainda carece de estudos mais específicos. Ainda sobre essas particularidades, as abelhas possuem uma relação de expectativa de vida/fertilidade diferente da maioria das outras espécies de insetos. Para essa maioria, quanto mais responsável pela fertilização, menor é a expectativa de vida do indivíduo. Já as abelhas que se comportam de forma contrária, as operárias, que não têm responsabilidade de reprodução, apresentam um ciclo de vida de 3 a 6 semanas, enquanto as rainhas, que se dedicam quase que exclusivamente à reprodução, vivem por até 3 anos. Essa condição ainda é pouco elucidada, principalmente por algo que chama atenção, que é o fato de

ambas – operárias e rainhas – se desenvolverem de um mesmo genoma (H. Shi et al., 2018).

Diante desses fatos, vários estudos têm buscado explicar o porquê dessas diferenças, primeiramente tentando considerar as questões genéticas. Diversas ferramentas de análise biomolecular vêm sendo utilizadas para tentar elucidar os mecanismos que estão por trás desses sistemas tão diferentes. Uma dessas ferramentas é a análise de transcritoma. Esse tipo de análise considera todos os transcritos do genoma de determinado indivíduo, isso permite analisar os diferentes padrões de expressão gênica de um organismo em diferentes situações, estágios de desenvolvimento, exposição a determinados ambientes, dentre outras possibilidades.

Em meio a análises comparativas de transcritomas de diferentes indivíduos, de diferentes espécies, abelhas e formigas por exemplo, pesquisadores conseguiram correlacionar dois genes que podem ser peças chave no envelhecimento das abelhas, o gene da Vg, e o gene do HJ (Korb et al., 2021).

O vitelo é o material presente nos ovos e que é a principal fonte de alimento durante o desenvolvimento do indivíduo após a fecundação. Por meio da vitelogênese, vários componentes são colocados à disposição durante a embriogênese, onde uma das principais proteínas componentes do vitelo é a Vg, sendo fonte de aminoácidos, carboidratos e lipídeos essenciais (Masuda & Oliveira, 1985). Trata-se de uma glicoproteína, produzida no corpo gorduroso do inseto sob forma de Vg, a mesma é transportada e acumulada na hemolinfa, à partir dali, é sequestrada para os ovários e ovócitos, onde sofre modificações e passa a vitelina, que vai ser de fato integrante do vitelo (Imperatriz-Fonseca et al., 2012). Sua correlação com o estágio de desenvolvimento em abelhas torna a Vg um alvo de extremo interesse para pesquisas. Seus índices se diferenciam tanto pela casta, quanto pela idade do indivíduo, sendo que as rainhas apresentam uma titulação aproximadamente 20% maior dessa proteína do que as operárias, que ainda vão sofrer com um decréscimo ao longo da vida. A produção de Vg aumenta logo após o estágio de pulpa, aumentando os níveis sanguíneos. O maior nível é observado em abelhas mães de 7 a 10 dias de idade, mas começam a cair logo que esses indivíduos começam a busca por alimento. Essa característica permite às abelhas mães utilizar a Vg como fonte de aminoácidos para a síntese de geleia real, principal alimento das larvas e da rainha (Ament et al., 2012).

Postulado pela primeira vez em 1934, por Wigglesworth, o HJ é um hormônio sesquiterpenóide, envolvido em diversos processos biológicos em artrópodes, com ação

no controle de diversas vias relacionadas ao desenvolvimento e reprodução, como por exemplo na muda, crescimento e maturação das gônadas (Qu et al., 2018). Tem como precursor o ácido farnesoico, e é produzido e secretado pela glândula endócrina corpora allata, por uma via bioquímica que difere entre as espécies (Qu et al., 2018).

A elucidação dos processos que envolvem o desenvolvimento nas abelhas há décadas chama atenção, principalmente pela complexidade dos mecanismos. Há um controle muito rígido envolvido, com mudanças drásticas e rápidas nas operárias devido ao seu curto tempo de vida. Alterações fisiológicas e na dieta são pontos chave nessas discussões, assim como a interação de moléculas como a Vitelogenina e o HJ. A titulação dessas duas em diferentes estágios de vida nas abelhas diz muito sobre esse controle, e proporcionam um vasto material de estudo.

Hartfelder e Engels correlacionaram em 1998 que as mudanças alimentares e fisiológicas das abelhas operárias acompanham um padrão de decaimento de Vg e aumento de HJ (Hartfelder & Engels, 1998). Esse padrão é chamado de modelo repressor duplo, onde altos níveis de Vg em operárias novas inibe a síntese de HJ mantendo níveis homeostáticos apenas (Guidugli et al., 2005). Durante a transição de abelha mãe para operária que faz a cobertura em busca de alimento, há uma inversão nessa relação Vg/HJ, os níveis de HJ voltam a aumentar, enquanto a expressão de Vg é inibida (Elekonich et al., 2003), esse ciclo de controle e influência entre Vg e HJ responde alguns questionamentos sobre essa transição das operárias entre abelha cuidadora, e abelha viajante. Muito ainda necessita ser tratado, principalmente pelo próprio comportamento de Vg e HJ nas diferentes espécies, dentro das abelhas por exemplo, HJ funciona como um hormônio gonadotrófico para o gênero Bombus, mas não em Apis (Shpigler et al., 2020).

2.4 microRNAs

miRNAs são pequenos RNAs de aproximadamente 22 nucleotídeos, endógenos, de fita simples, não codificantes, envolvidos no controle da expressão gênica a nível pós-transcricional (W. Liu & Wang, 2019). Esse controle é exercido pelo pareamento da sequência de miRNA com o RNAm, provocando a degradação desse RNAm ou um impedimento histérico, de forma que o mesmo não possa interagir corretamente com o ribossomo (Lucas & Raikhel, 2013). São uma maquinaria altamente específica, que exercem um controle refinado em diversos processos biológicos como desenvolvimento

embrionário, diferenciação tecidual, proliferação celular e morfogênese (Behura & Whitfield, 2010). A biogêneses de miRNA é um evento de vários passos, até que o mesmo esteja apto a desempenhar sua função (Lucas & Raikhel, 2013). À priori, no núcleo, o transcrito de miRNA se dobra em uma estrutura de hairpin, sendo essa sua estrutura primária (pri-miRNA); em seguida há a primeira fase processiva onde o pri-miRNA sofre ação de uma RNAse III (Drosha), formando um miRNA precursor (pre-miRNA). O mesmo é exportado ao citoplasma onde novamente uma RNAse III (Dicer) faz o processamento formando uma dupla fita de miRNA, numa estrutura miRNA-miRNA. Esse duplex de miRNA é incorporado a um complexo proteico chamado RISC (do inglês, RNA induced silencing complex), em que a proteína Argonauta é uma das principais componentes. Dentro do complexo RISC o duplex de miRNA é separado e apenas uma das fitas será dita como o miRNA maduro e irá desempenhar sua função (Cheloufi et al., 2010).

Através de sua função de controle de expressão gênica, os miRNAs já tem sido citados como importantes reguladores de diversas funções em abelhas, como a diferenciação de castas, diferenciação entre operárias responsáveis pela coleta de alimento e "operárias dançarinas", e ativação de ovários (Ashby et al., 2016). Liu et al, em 2019, descreveram um padrão de expressão gênica comparando abelhas macho, rainha e operárias, onde 19 miRNAs tiveram uma notória diferença do padrão de expressão (M. Liu et al., 2019). Já Chen et al, 2017, conseguiram estreitar a relação de miRNAs influenciando na postura de ovos e na ativação dos ovários, onde 81 miRNAs apresentaram diferentes perfis de expressão em rainhas virgens e rainhas já em postura de ovos, e 17 miRNAs com diferentes expressões em condições de inibição de postura de ovos e postura de ovos normal (X. Chen et al., 2017).

De maneira geral em insetos, miRNAs tem mostrado uma estreita relação de controle de Vg, logo, alterando a maturação de oócitos e a fertilidade de modo geral. A ausência de miRNA-184 em Drosophila levou a perda de produção de ovos (Iovino et al., 2009), assim como a depleção de proteínas da via, Dicer e Argonauta, culminaram na diminuição dos índices de Vg e o comprometimento da maturação de oócitos em gafanhotos (Song et al., 2013). Ainda nesse contexto, os miRNAs let-7 e miRNA-278 apresentaram como alvo um transcrito, que quando reprimido mostrou um decaimento de Vg e bloqueio na maturação de oócitos (Song et al., 2018). Neste mesmo estudo foi observado também que a titulação de HJ aumenta se houver uma diminuição na expressão de let-7 e miRNA-278, fazendo com que HJ seja um reativador da via de

vitelogênese, já que sua superexpressão diminui a expressão dos miRNAs citados. Em um estudo anterior, Nunes et al (2013), mostraram uma diminuição na expressão em let-7 e miRNA-281 em abelhas operárias *knockdown* para Vg (Nunes et al., 2013).

Kapheim e colaboradores publicaram em 2020 um estudo comparativo da expressão de miRNAs em cérebros de 12 diferentes espécies de abelhas, eussociais e solitárias, onde puderam observar que aproximadamente 35% dos miRNAs identificados eram espécie específicos. Além disso, o miRNA-305 foi identificado apenas nas espécies sociais, estando ausente nas solitárias (Kapheim et al., 2020).

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ARTIGO

New insights into bee microRNAs: computational identification and characterization of pathway genes, conserved microRNAs and their targets.

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ABSTRACT:

MicroRNAs are noncoding RNA sequences that control and regulate post-transcriptional gene expression. We performed a genome-wide computational prediction of miRNAs to improve the understanding of miRNA observation and function in eight species of bees. As an initial step, genome scanning predicted hundreds of conserved miRNAs in the subject species. Applying an optimized algorithm, we located precursors of miRNAs in the genome of *Apis mellifera*, *Bombus impatiens*, *Duforea novaengliae*, *Eufrisea mexicana*, *Habropoda laboriosa*, *Lasioglossum albipes*, *Megachile rotundata,* and *Melipona quadrifasciata*. Some of these miRNAs were selected for deep structure analysis. Furthermore, proteins Argonaute, Dicer and Drosha, key proteins in miRNA biogenesis pathway, were emphatically addressed and analyzed aiming the comparison between subject bees' species and ortologues, focusing the verification of the conservation level of the active site amino acids residues, the conservation of key domains, and phylogenetic analysis. Among all functional proteins in bees, Vitellogenin (Vg) stands out due its role in embryo development, being the nutritional reserve in nonmammalian vertebrates. We found seven potential miRNAs that effectively work in Vg control based on miRNA target identification. Our results will open up a new avenue for studying miRNAs in bee biology.

KEYWORDS: Bioinformatics; in-silico analysis; microRNAs, bees.

1 Introduction

Bees are the most abundant pollinators in agriculture, as they visit more than 90% of the 107 main crops (Klein et al., 2007). 70% of fruits and seeds are produced with better quantity and/or quality when properly pollinated, displaying bees' economic impact on agriculture (Kerr et al, 1996; Vit et al., 2018). Pollination contributes to the maintenance and genetic variability of native plant populations that support biodiversity and ecosystem functions, ensuring a reliable and diversified supply of fruits, seeds, and honey, among others (Costanza et al., 2017).

Divided into castes such as workers and queen, bees are haplodiploid organisms. Haploid individuals are male and diploid individuals are female; therefore, females are derived from fertilized eggs and males from unfertilized eggs (Bull, 1987; Cook, 1993; Dzierzon, 1845). For *Apis mellifera,* the difference in the quality of food offered to young larvae triggers specific developmental pathways, producing female phenotypes: queens or workers (MacEdo et al., 2016). Royal jelly has been revealed to have the potential to stimulate larvae to develop into a queen. (Kamakura, 2011). Many studies have focused on gene expression profiles to identify candidate genes that affect bee ovarian development and activation (Vergoz et al., 2012). Identification of genes expressed in bee ovaries brought advances in understanding gene regulation mechanisms associated with ovarian phenotypic differences (Lago et al., 2016; Y. Y. Shi et al., 2015). Factors such as circular RNAs (circRNAs) play roles in a potentially complex network that regulates ovarian physiology and activation (X. Chen et al., 2019).

MiRNAs are non-coding RNAs of approximately 22 nucleotides which control gene expression at the post-transcriptional level (W. Liu & Wang, 2019). This control is exercised by the miRNA sequence pairing with the mRNA, degrading it or causing steric hindrance, impeding its transcription (Lucas & Raikhel, 2013). As important as the study of miRNAs, their pathway genes are of paramount importance, where the proteins Argonaute, Dicer, and Drosha are at the center of crucial steps in miRNA processing and maturation (O'Brien et al., 2018).

Since the first publication describing the discover of miRNAs (R. C. Lee et al., 1993), this molecule has been tirelessly studied. As far as the concept and discovery of functions played was increased, there was a need to establish the relationship of miRNAs, mRNAs and key proteins involved in as many biological mechanisms as possible. Later, miRNAs were taken as critical in health animal development, specifically in bees, miRNAs have already been quoted as important regulators of various behaviors, such as the variety of functions performed by workers through their lifespan (Ashby et al., 2016), and more, evidence indicates that miRNAs are also involved in the regulation of insect metamorphosis and wing development (Belles, 2017; Lozano et al., 2015). In previous studies, several miRNAs have been correlated to play important roles in the modulation of tissue patterns, cell differentiation, ovarian development, and caste determination in bees. However, the investigation of the critical activity of lncRNAs, miRNAs, and the lncRNA-miRNA-mRNA network in queen bee oviposition is still needed. For example, *ame-miR-184* and *ame-miR-315*, have been identified and described as involved in a caste-independent ovarian activity in queens and workers (Ashby et al., 2016; MacEdo et al., 2016).

An important gene involved in development among vertebrates is Vitellogenin (Vtg). This gene encodes for Vitellogenin (Vg) family protein, a group of polypeptides that are precursors of yolk proteins,

an important source of energy in embryo development for oviparous and ovoviviparous vertebrates (Carducci et al., 2019).

To improve the understanding of the characteristics and function of the miRNA in bees, in silico analyses of genes involved in the miRNA biogenesis pathway and precursor and mature miRNAs were performed in eight different species of bees in order to identify and characterize these molecules. This study aimed to identify and characterize, by in silico methods, mature miRNAs, their respective precursors, their possible targets – more specifically for Vitellogenin – and the genes involved in their processing pathway in the genome of the species: *Apis mellifera*, *Bombus impatiens*, *Dufourea novaeangliae*, *Eufriesea mexicana*, *Habropoda laboriosa*, *Lasioglossum albipes*, *Melipona quadrifasciata*, and *Megachile rotundata*. Our results will open a new path to the study of miRNA in the biology of bees, helping to understand similarities and differences that justify morphological, physiological, and behavioral modifications

2 Material and methods

2.1 miRNA pathway: Gene prediction and proteins characterization

The putative proteins involved in the miRNA processing pathway were identified and selected by mining bee sequences from the basic local alignment search tool BLAST (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/), BLASTp algorithm, and protein sequence from *D. melanogaster*. To obtain a complete set of putative orthologous proteins to compare with bee proteins, invertebrate species reference proteins were searched at the NCBI using the BLASTp tool and non-redundant database.

2.1.1. Phylogenetic analysis, multiple alignments, and active site analysis of miRNA pathway proteins

Analyzes of protein families, domains, and active sites were performed using PFAM online tool (v 31.0) (http://pfam.xfam.org/) and Conserved Domains Database (CDD) (http://www.ncbi.nlm.nih.gov/cdd/). The protein sequences of bees and orthologous species were used to perform multiple sequence alignments using ClustalX (v 2.1) (http://www.clustal.org/) with default settings (available at http://www.clustal.org/clustal2/) (Larkin et al., 2007).

Phylogenetic trees were inferred using the Neighbor-Joining method, and sequence divergence was estimated using the Jones – Taylor – Thornton model (Saitou & Nei, 1987). Statistical reliability of internal branches was evaluated using 2000 bootstrap replicates. The Phylogenetic molecular analyzes were conducted using MEGA X software (Tamura et al., 2011). Sequence logos were generated using WebLogo 2.8.2 (http://weblogo.berkeley.edu/logo.cgi).

2.2 Prediction and characterization of both precursor and mature miRNAs

The genome of all eight species was retrieved from BeeBase – Hymenoptera Genome Database (https://hymenoptera.elsiklab.missouri.edu/beebase), utilizing the latest version of each one provided by

the Ten Bees Project (Kapheim et al., 2015). A robust algorithm established by de Souza Gomes et al (2011) was applied to predict precursors and mature miRNAs. This tool considers the optimum conditions that are expected in a sequence to show potential hairpin formation or precursor miRNA similarities. The algorithm selects sequences that cover some filters indispensable to predict putative miRNA precursors, and they are GC content, MFE, and different levels of homology (miRNAs previously described to be closer species, with protein-coding regions, repetitive regions, and non-coding RNAs).

The sequences that were selected by the algorithm advanced in our analysis with the prediction of thermodynamics features and secondary structure, where it was used RNAFold (Vienna RNA Package) (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) (Lorenz et al., 2011) and a homemade Perl script. These tools were applied to the cluster and the individual miRNAs.

miRNA cluster 71/2 was predicted by checking the start and end position of the miRNAs that belonged to this structure, observing if it was located in the limit of 10k nucleotides of distance.

2.2.1 miRNA alignment and phylogenetic analysis

Pre-miRNA sequences from bees and orthologues were submitted to a multiple sequence alignment into ClustalX2 software (Larkin et al., 2007), following the parameters, 22.50 of gap opening and 0.83 of gap extension. On the other hand, pre-miRNA sequences from bees and orthologues were used to conduct phylogenetic analysis, using the MEGAX software (Kumar et al., 2018) and Neighbor-Joining method with the parameters adjusted: Bootstrap method was applied, a consensus tree with 5000 replications, and Kimura 2 parameters as substitution model in the evolutionary distance calculation (Saitou & Nei, 1987).

2.3 miRNA target prediction

Putative gene targets searching in *A. mellifera*, considered mostly 3' UTR. 3' UTR sequences were retrieved from GFF data deposited in BeeBase. The sequences were analyzed in two steps. Firstly, based on Free Energy and Conservation, we conducted this analysis in miRanda software (John et al., 2004). Next, an analysis comprising the most consistent options considering the miRNA: mRNA energy of hybridization was performed (Krüger & Rehmsmeier, 2006).

2.4 Statistical analysis

Basic descriptive analyses followed by normality tests (Kolmogorov-Smirnov) were performed, and it was verified that the MFE, MFEI, and AMFE variables have a non-normal behavior. Therefore, the comparison between the species of these variables was made based on the differences between the medians by the Kruskall-Walis method between independent samples. In this case, the Kruskal-Wallis test with $\alpha = 0.05$ determinate if one or more of the groups are significantly different (Journal et al., 2017; Kruskal & Wallis, 1952).

3. Results and Discussion

3.1 miRNA biogenesis pathway in bee

MiRNA biogenesis is a pathway with several steps. It demands a considerable number of proteins to be able to perform its function, including: AGO2, DGCR8, DICER, DROSHA, TRABP2, RAN, and XPO5 (Lucas & Raikhel, 2013). At first, in the nucleus, the miRNA transcript folds into a hairpin structure, which is its primary structure, the primary miRNA. The first processive phase starts, and the primary miRNA is cleaved by Drosha, forming the miRNA precursor (Han et al., 2004).

The precursor is exported to the cytoplasm, where Dicer cleaves it and removes the loop from the structure, forming a miRNA-miRNA duplex. This miRNA duplex is incorporated into a protein complex called RISC (RNA-induced silencing complex), in which the Argonaute protein is one of the main components. Within the RISC complex, the miRNA duplex is dissociated, and only one of the strands is used as the mature miRNA (Cheloufi et al., 2010).

The predicted proteomes, available on Beebase (https://hymenoptera.elsiklab.missouri.edu/beebase) - with the corresponding versions used: *Apis mellifera* – 4.5, *Bombus impatiens* – 1.0, *Megachile rotundata* – 1.1, *Dufourea novaeangliae* – 1.1, *Eufriesea Mexicana* – 1.1, *Habropoda laboriosa* – 1.2, *Lasioglossum albipes* – 5.42 and *Melipona quadrifasciata* – 1.1 -, enabled the identification of nine proteins involved in the miRNA pathway- DROSHA, DICER, ARGONAUTE, RANBP21, PASHA, TUDOR, FMR1, VIG2 and LOQUACIOUS (Supplementary data I – Table I). Among them, two families of proteins were chosen to further analysis due-to their main role in miRNA processing and maturation: Argonaute and RNAse III proteins (DICER and DROSHA). According to homology to *D. melanogaster*, 41 putative proteins were identified based on protein size, domains conservation, and distribution. On average, each species displayed five proteins, three RNAse III, and two Argonaute.

3.1.1 DROSHA

DROSHA is a nuclear enzyme that has a dsRNA (double-stranded RNA) binding domain and two RNase III domains, each one involved in the cleavage of the pri-miRNA arms (Y. Lee et al., 2003). The Hairpin cleavage performed by DROSHA alongside DGCR8 in the miRNA needs to be a precise process because it defines the terminus of the miRNA (Han et al., 2006).

In the analysis, eight putative Drosha proteins were identified - GB49096, BIMP25827, Dnov10175, Emex08506, Hlab12928, Lalb_00686, Mqua07049, and Mrot00920 -. All of them presented the three required domains: Ribonuclease III-like, Ribonuclease III, and Double-stranded RNA binding motif (DSRM). The domain positions are displayed in Supplementary data II - table I, while their distribution is in Fig. 1.

The catalytic residues of the Riboc I domain showed the active site catalytic motif (EDNE): glutamic acid (E), aspartate (D), and asparagine (N). The second Riboc presented the active site catalytic motif (EDDE): glutamic acid (E), and aspartate (D) (Fig. 2). The two conserved Ribonuclease III domains identified in the putative DROSHA showed a highly conserved active site compared to their orthologs. The positions for each amino acid are in Supplementary data II – Table I. The phylogenetic analysis of DROSHA

putative proteins displayed the evolutionary relationship with their orthologs and paralogues. All eight bee putative proteins clustered within the arthropods clade on the Drosha (Fig. 3).

3.1.2. DICER

DICER participates in processing RNA precursor in the cytoplasm by cleaving it near the terminal portion of the loop to release the miRNA duplex. Most animal species contain only one DICER, such as mammals and nematodes; however, some species have several homologs with different functions. *D. melanogaster* has two DICER; DICER-1 is responsible for the production of miRNA, and DICER-2 is involved in the synthesis of other small RNAs (Y. Lee et al., 2004).

In the analysis, sixteen proteins were analyzed and identified as DICER - GB48923, GB44595, BIMP24576, BIMP15231, Dnov01654, Dnov12532, Emex00397, Emex09608, Hlab00411, Hlab03512, Lalb_01265, Lalb_01487 Mqua02257, Mqua14519, Mrot05715, and Mrot13039 -. The following seven domains were localized in the putative Dicer proteins, Ribonuclease III, Ribonuclease III, PAZ, Dicer dimerization, Type III restriction enzyme subunit, Helicase conserved C-terminal and DSRM (Fig 1, and Supplementary data II - Table II). One of them showed an unexpected domain, Lalb_01265, a centrosomal colon cancer autoantigen domain, which could infer an assembly genome error.

After aligning with its homologous protein, NP_524453.1, its initial codon appears to be 704Gln, which would make the start of the first domain, Type III restriction enzyme - res subunit, the 18Arg, which corroborates with the results of other proteins, like GB48923, BIMP24576, Dnov01654, Emex00397, Hlab03512, Mqua14519, and Mrot05715.

The catalytic residues of Riboc I and II domain showed the active site catalytic motif (EDDE): glutamic acid (E), and aspartate (D). The positions for each amino acid are in Table II (Supplementary data II), and the Riboc I and II conserved domain analysis are in Fig. 2, and Supplementary data II - Table II. Only one of them, Hlab03512, did not present all motifs for Riboc II. This could mean the protein is truncated.

The phylogenetic analysis of DICER was done simultaneously with Drosha proteins. All bee proteins clustered within the arthropods clade on the Dicer clade (Fig. 3).

3.1.3. ARGONAUTE

The ARGONAUTE family of proteins is a large family of proteins of about 95 kDa that have two important subfamilies, AGO and PIWI. These subfamilies play important roles in non-coding RNA pathways. AGO proteins constitute the main protein of the RISC complex in miRNA and small interference RNA (siRNA). PIWI proteins are responsible for the RISC complex in RNAs that interact with the PIWI pathway (piRNA) (Carmell et al., 2002; Hutvagner & Simard, 2008).

The ARGONAUTE subfamilies show two highly conserved domains, the PIWI and PAZ domains, found in most organisms. The PAZ domain recognizes the 3' end of the attached small RNA, while the PIWI domain binds the target mRNA for cleavage (Wang et al., 2008). The PAZ domain is positioned at the N-terminal end of the protein, while the C-terminal region presents the PIWI domain. The arrangement of the domains is strategic since it can position the small RNA in its target gene. The PIWI domain has a catalytic site responsible for the cleavage of target messenger RNA (mRNA). This active site was composed

of three amino acid residues: DDH (Aspartate, Aspartate, and Histidine) or DDD (three Aspartates) (Kawamata et al., 2009; Kiriakidou et al., 2007).

Seventeen proteins were analyzed and identified as putative Argonaute proteins, GB48208, GB50955, BIMP23552, BIMP15146, Dnov12168, Dnov12686, Emex11192, Emex05584, Hlab06842, Hlab08547, Lalb_03345, Lalb_04947, Mqua07112, Mqua06975, Mrot02381, Mrot10140, and Mrot08389. These proteins identified six conserved domains: Piwi, Mid domain of argonaute, N-terminal domain of argonaute, PAZ, Argonaute linker one and Argonaute linker 2 (Fig 4, and Supplementary Data II, Table III). Although all of them presented the PIWI domain, three of them did not display the PAZ domain, GB50955, Hlab08547, and Mrot10140.

The putative proteins, Mrot10140 and Emex05584, presented a different size compareded to others, which could infer an assembly genome error. After aligning them with their homologous proteins, the start of Mrot10140 and the end of Emex05584 changed. The first codon of Mrot10140 is 1359Phe, and the last codon of Emex05584 is 838Pro.

The catalytic residues of the Piwi domain presented the active site catalytic motif (DDH): aspartate (D) and Histidine (H). The positions for each amino acid are in Supplementary Data II, and Table III, and the analysis of the Piwi domain is in **Erro! Fonte de referência não encontrada.**.

The phylogenetic analysis of Argonaute was made simultaneously with Piwi. All seventeen bee proteins clustered within the arthropods clade on the Argonaute clade (**Erro! Fonte de referência não encontrada.**).

3.2. Genome-wide scanning of miRNA in bee

3.2.1 Mature miRNA and their precursors in bee

To show that miRNA processing machinery is well conserved in bees, we sought to identify mature and precursor miRNA sequences, in addition to predicting and defining the nature of their putative target genes using public available databases on the genome of the species studied. Our results suggest that, in general, miRNAs demonstrated similar evolutionary patterns among the eight species studied and their orthologues.

Applying an optimized algorithm, 432 sequences of mature miRNAs (3p and 5p) with 329 precursor miRNAs in the genome of *A. mellifera* were identified. In *B. impatiens*, 321 sequences of mature miRNAs and 226 precursor miRNAs were established. For the species *D. novaengliae*, 288 mature miRNAs and 207 precursor miRNAs were identified; In *Eufrisea mexicana*, 235 mature miRNAs and 163 precursor miRNAs were determined; *H. laboriosa* revealed 298 mature miRNAs and 221 precursor miRNAs. For the bee *L. albipes*, 275 sequences of mature miRNAs and 197 precursor miRNAs were identified. In *M. rotundata* 309 mature miRNAs and 210 precursor miRNAs. Finally, in the genome of the *M. quadrifasciata* species, 282 mature miRNAs and 200 precursor miRNAs were characterized (Supplementary data III, Tables I - VIII).

In the middle of the 329 precursor miRNAs in the genome of *A. mellifera* identified in our study, 124 were identified for the first time; from these new sequences described in *A. mellifera* such mir-1187 family (ame-*mir-1187-1,* ame-*mir-1187-1,* ame-*mir-1187-3,* ame-*mir-1187-4*), mir-1277 family (ame-*mir-*

1277-1, ame-*mir-1277-2,* and ame-*mir-1277-3*), mir-466i family (ame-*mir-466i-1,* ame-*mir-466i-2,* ame-*mir-466i-3,* ame-*mir-466i-4,* and ame-*mir-466i-5*) among others.

Among the miRNA families identified, 24 pre-miRNA precursor sequences occurred in all eight bee species evaluated, namely: *mir-10, mir-184, mir-210-1, mir-210-2, mir-263b, mir-2b-2, mir-3049, mir-305, mir-316, mir-33a-1, mir-3478, mir-3529, mir-3719, mir-3783, mir-3786, mir -466i-1, mir-466i-2, mir-6001, mir-6060, mir-6067, mir-9-2, mir-9-3, mir-993b and mir-2765*. Other pre-miRNAs were also highly prevalent and distributed across species. The following miRNAs were found in at least seven species: *iab-4, let-7-2, mir-124b, mir-125-2, mir-133, mir-137, mir-14, mir-1b, mir-193, mir-1b,, mir-277, mir-279-3, mir-2b-1, mir-315-2, mir-2b-3, mir-2b-4, mir-375, mir-6003, mir-6012, mir-6037, mir-6038, mir-6040, mir-6065, mir-750, mir-7c, mir-927b, mir-929, mir-92c, mir-932, mir-965, mir-981-2, mir-9869, mir-9882, mir-996, mir-iab, mir-317,* and *mir-71* (Supplementary data IV - Table I and II).

Some studies have identified miRNAs in the bee sequences, but so far, no attempt has been made to systematically identify the miRNA repertoire in the genomic sequence of eight species concurrently. Gu et al (2010), reported 267 miRNAs in four developmental stages (egg, larva, pupa, and adult) and pointed to *ame-miR-281* and *ame-miR-33* as the most frequently sequenced miRNAs. F. Liu et al (2012), in turn, found 107 mature miRNAs when evaluating the miRNA expression profile comparatively between nurses and foragers, reporting that the five most expressed miRNAs in both nurses and foragers were ame-*miR-1, ame-miR-276, ame-miR-184, ame-miR-996,* and *ame-miR-275*; a*me-miR-31a,* and *ame-mir-13b* were further validated using quantitative PCR assays with higher expression rates in both castes.

MacEdo et al (2016), using a sequenced bee ovary transcriptome library, comparatively studied miRNAs expressed in the ovaries of worker and queen bees (virgin and mated), concluding that these molecules are deeply involved in the determination of caste-independent reproductive status. The authors indicated that 19 miRNAs showed dynamic expression in both active and inactive ovaries, establishing these miRNAs (*miR -1, miR -31a, miR -13b, miR -125, Let-7 RNA, miR -100, miR -276, miR-12, miR -263a, miR -306, miR -317, miR -92a, and miR -9a*) are useful in assessing the reproductive status. M. Liu et al (2019) analyzed the expression of miRNAs in the heads of three castes of the *Bombus lantschouensis* bee and identified 364 known miRNAs and 89 new miRNAs.

3.2.2. MFE, MFEI and AMFE analysis

All identified miRNAs were analyzed for their structural and thermodynamic characteristics. The miRNA precursors of the eight species studied showed an MFE, AMFE, and MFEI with means shown in Supplementary data V. The studied bees exhibited an MFE with an average of -33.568 kcal/mol. MFE is an important feature used to distinguish miRNA precursors and, therefore, used to distinguish real and pseudo sequences. A miRNA precursor molecule is stable and can generate mature miRNA with an MFE value of -20 kcal/mol. (Zhao et al., 2010). Li et al (2013) systematically analyzed, using bioinformatics tools, characteristics of pre-miRNAs in 24 insect species and established an average MFE of - 33.07 kcal/mol for these species. Individually, they demonstrated MFE in these insects, such as *A. mellifera* (MFE: -35.94±9.84 kcal/mol), *Culex quinquefasciatus* (MFE: - 34.25±7.51 kcal/mol), and *Drosophila melanogaster* (MFE: –33±10.99 kcal/mol) corroborating our results.

MFEI values with a mean value of 0,81 kcal/mol were obtained in our study, and above 0.85 kcal/mol were suggested for potential precursors of miRNAs, differentiating them from other non-coding RNAs. B. H. Zhang et al (2006) developed this term called minimum index energy analysis (MFEI) to detect different types of RNA in plants showing that RNA sequences with average AMFE greater than 0.85 kcal/mol are more likely to be real miRNA suggesting that MFEI can be easily used to distinguish miRNA of other non-coding RNAs, like mRNA, tRNAs, or rRNAs. Employing this concept, J. Li et al (2013), described characteristics of pre-miRNA from 24 insect species and observed that most species had mean MFEIs greater than 0.85 kcal/mol as observed in the species: *A. mellifera* (MFEI: 0.81 kcal/mol) and *D. melanogaster* (MFEI: 0.83 kcal/mol), supporting our result.

Statistical analysis was applied to this data, aiming to ascertain if the parameters MFE, AMFE, and MFEI, were divergent among our species. According to the data, the results from Kruskal-Wallis test did not indicate that one or more groups are significantly different for any of the parameters, considering the following p-value, MFE p-value = 0.286 > 0.05, AMFE p-value = 0.201 > 0.05 and MFEI p-value = 0.113 > 0.05 (Fig 7).

3.3 miRNAs characterization

Some miRNAs have been hardest studied due to their pivotal role in essential biological processes. Based on such importance and relevance, among the miRNA families described above, four miRNAs, mir-1b, mir-2b, mir-283, and mir-927, stood out for further and detailed characterization. The conservation of their sequences, and phylogenetic distributions were analyzed for each of them. Critically, the targets of miRNAs were also sought to identify the biological processes in which these new miRNAs were involved.

3.3.1. miR-1b

Nine mir-1b precursors were identified along with seventeen mature miRNAs distributed across the eight species of bee. miR-1b is a member of the miR-1 family, and its sequence is highly homologous to miR-1 (Y. pu Liu et al., 2018). The align comprising miR-1b with their orthologues demonstrated high conservation between miR-1b sequences found in the bees and their orthologues (Fig. 8), further evidenced by the secondary structure analysis, which also showed great conservation between the bee miRNAs and their orthologues (Fig. 9). The phylogenetic analysis generated a tree divided in three well-defined clades, which were identified as Arthropoda, Chordata, and Nematoda. All sequences were grouped according to the distribution found in the animal tree of life, with the bees being mostly close to each other within the arthropod clade (Fig 10).

The presence of miR-1b has been reported in other arthropods, such as *Manduca sexta* (X. Zhang et al., 2014), *Bombyx mori* (S. Liu et al., 2010), and *Dinoponera quadriceps* (Patalano et al., 2015), corroborating with the sequences identified in the bees. Additionally, miR-1b has been identified and studied in several different organisms, such as chickens, rats, and schistosomes (J. Li et al., 2020; Y. pu Liu et al., 2018; Zhu et al., 2016).

The molecular mechanisms of miR-1b in bees have not yet been properly investigated. However, a study on bee ovaries reported that miR-1 is associated with ovarian activation in bees, being highly expressed in this organ under certain reproductive conditions (MacEdo et al., 2016). Li et al., (2020) studied

the effects of miR-1b (gga-miR-1b-3p) in chicken ovaries and found an abundance of miR-1b-3p in follicular theca cells, indicating its involvement in regulating ovarian functions. This is indicative that, in bees, miR-1b might also have a role in the regulation of ovarian functions.

A study on Wistar rats showed that miR-1b targets Krüppel-like factor 7 (KLF7) (X. Li et al., 2021). KLF7 regulates the differentiation and proliferation of neuronal cells (Caiazzo et al., 2010). It also has been shown that KLF7 plays an important role in peripheral nerve injury recovery by promoting growth, axonal regeneration, and sprouting in neurons (X. Li et al., 2021). In rats, miR-1b is a regulator of neuron proliferation and regeneration. Additionally, KLF7 orthologues have been identified in *D. melanogaster* and showed great similarity with mammalian KLF7 in its sequence identity and many conserved features (De Graeve et al., 2003). Although the function of KLF7 orthologues has not been completely elucidated, a decrease in its activity has great negative impacts on *D. melanogaster* development, meaning this transcription factor is extremely important for early embryogenesis (De Graeve et al., 2003). This evidence suggests that miR-1b could regulate embryonic development in insects.

3.3.2. mir-2b family

miR-2 is one of the most important families of miRNAs distributed among invertebrates (Marco et al., 2012). Their presence in such clades gives this family an important variation of functions, and the number of genes is variable according to each species. The largest distribution is in *D. melanogaster*, where we have eight members of this family: *mir-2a-1*, *mir-2a-2*, *mir 2b-1*, *mir-2b-2*, *mir-2c*, *mir-13a*, *mir-13b-1*, and *mir-13b-2* (Ruby et al., 2007). In *S. haematobium* were found five precursors of this family (Cardoso et al., 2020). Using our pipeline, representatives of *miR-2* family were found in all eight bee species, totaling 48 precursors and 75 mature miRNAs (*A. mellifera* with six precursors, *B. impatiens* with six precursors, *D. novaenglie* with seven precursors, *E. mexicana* with seven precursors, *H. laboriosa* with three precursors, *L. albipes* with seven precursors, *M. rotundata* with six precursors and *M. quadrifasciata* with six precursors. MacEdo et al (2016) also reported six precursors in *A. mellifera*, corroborating with our results, notwithstanding all the others are being presented for the first time. Until this date, miRBase provided 42 representatives for miR-2 in different species, where, only *A. melifera* is related.

The predicted *miR-2* family was confronted against those in miRBase, based on general structure and thermodynamic features. *A. mellifera* was used as a model, once it was the most studied among the subjective species. Commonly, miRNAs belonging to the same family present themselves in clusters, at most 10 kb distant from each other. Although *miR-71* clusters itself with *miR-2* (De Souza Gomes et al., 2013), the target of our analysis was *miR-2b*. Therefore, only *miR-71* secondary structure is present in order to represent the *miR-71/2 cluster*. The genomic localization of *miR-2* family representatives and *miR*-71, showed that the majority of our species corroborate with this information. Only *E. mexicana* showed two different clusterizations between *miR-2b/13a* and *miR-13b-2/71*. In *H. laboriosa miR-13* was not found, and for *L. albipes miR-71* was not found. Therefore, for those species, the cluster was not complete (Supplementary data VI). Fig. 11 shows this *mir-71/2 cluster* organization, the conservation can be seen through the secondary structure, even in the species in which the cluster is not complete. It was possible to observe a hundred percent of nucleotide conservation in the seed region, from the 2 - 8 nt, in both 3p and 5p (Fig. 12a, b, and c). Fig. 13a, 13b, and 13c represent the phylogenetic analysis with a variety of

orthologues. Was possible perceive that the clades organization are in according to the tree of life and obey the structuring proposed by Marco et al (2012).

Due to their large distribution in invertebrates, important studies have been published linking *miR-2* family to countless functions. In *A. aegypti,* miR-2b was introduced as one of the mechanisms involved in the defense against virus infection, like *Chikungunya virus* (Dubey et al., 2017). *Plutella xylostella* is a pest that causes catastrophic damage to cruciferous plants. This occurs because *P. xylostella* has evolved, over time, resistance to synthetic chemical insecticides of any class. In a recent study, Etebari et al (2018), showed that *mir-2b-3p* is involved in cytochrome P450 regulation (CYP9F2;), which is one of the mechanisms used by *P. xylostella* in insecticide resistance. Similarly, Hong et al (2014) showed different patterns in *mir-2b-3p* and *miR-13* expression in a comparison between both deltamethrin susceptible and resistant *Culex pipiens pallens.*

Drosophila melanogaster is a species used as a model organism. *mir-2b-3p* has been described in *D. melanogaster* with some importance in sleep coordination (Goodwin et al., 2018). *miR-2* family is also associated with embryo development. Boutla described development defects in the head and posterior abdominal segments, after inactivation of *mir-2a* and *mir-13a* in *D. melanogaster* embryos (Boutla et al., 2003).

A global analysis of enriched GO terms linked *miR-2* family members with 675 genes in *D. melanogaster*, involved in a range of functions, such as neurogenesis, cell differentiation, nervous system development, and more. In the same study, 979 genes were determined as *miR-2* targets, been involved in anatomical structure morphogenesis, generation of neurons, organ morphogenesis, and more (Marco et al., 2012). Ling and collaborators proposed the importance of *miR-2* in normal wing development. They demonstrated that *miR-2* has *fng* and *awd* genes as targets, and alterations in *miR-2*, like over-expression, produced unnaturally aberrant wings in adults (Ling et al., 2015).

Last few years, the community had been noticing colony losses of honeybees worldwide. There are a lot of possible explanations for this phenomenon. One of them is the infection of colonies by different microorganisms such as bacteria, viruses, and parasites. *Nosema* is a microsporidian parasite responsible for the infection of young adult bees that can disable those infected from producing food or royal jelly. Honeybees have microRNAs as apparatus against infection. In *N. ceranae* infection, there are significant modifications in the pattern of miRNA expression, guiding to a differential expression of key miRNAs, among then *miR-2b*. They are correlated with pathways such as oxidative phosphorylation, glycan degradation, and biosynthesis of antibiotics (Huang et al., 2015). Following this route, it was tested genetic variants that give honeybees subspecies some level of resistance to *Nosema* infection. One of the microsatellite loci evaluated is located in the chromosome 1.1 region, the same position as *miR-2b* (Ostroverkhova, 2021).

3.3.3. miR-283

In the analysis, the miRNA-283 family was identified in all bee species. There were eight precursors and sixteen mature miRNAs. Only *ame-mi-283* was already described, being available on miRbase. *A. mellifera* miRNA was used as a model to analyze the others, comparing their sequence, structure and thermodynamic features. All of them presented high conservation compared to their ortholog

in both primary and secondary structures analysis (Fig. 14 and 15), which is important to draw attention to the 3p and 5p seed region of the miRNA, where we got 100% of nucleotide conservation. The phylogenetic tree is divided into three clades: Lepidoptera, Branchiopoda, Diptera, and Hymenoptera. In the analysis of *miR-283* relationship with their orthologs and paralogues, all eight bee *miR-283* gathered within the Hymenoptera clade, being mostly close to each other, according to the tree of life (Fig. 16).

The *miR-283* family was found in several other organisms belonging to the superorder Endopterygota, including *D. melanogaster* (Stark et al., 2007), *Daphnia pulex* (Wheeler et al., 2009) and *Tribolium castaneum* (Marco et al., 2010). In other studies, miR-283 was also identified in the nematode phylum, *Brugia malayi* (Poole et al., 2014).

Although the expression of the *miRNA-283* has been validated experimentally, its biological functions and mechanisms have not been elucidated yet, especially for species that are not used as models. However, some studies bring up an initial understanding, linking it to multiple functions.

Research on *D. melanogaster* points *to a multifunction miR-283, linked to a male-biased function and* the immune system. Marco (2014) phylogenetic study indicated that the *miR-283* is an old and least sex-biased molecule that did not emerge within the Drosophila lineage. Furthermore, the same researcher in 2015 concluded that this miRNA might contribute to the destabilization of maternal transcripts, but probably as zygotic microRNAs. In the immune system, *miR-283* targets PGRP-LC and imd. Both receptors are involved in detecting components of Gram-negative bacterial cell walls (Fullaondo & Lee, 2012). Bhattacharya et al (2021) also reported the potential of *miR-283* action on the Drosophila immune system, this time targeting the 30 untranslated regions (30UTR) of the DNMT2 gene, downregulating its expression. This gene is an essential determinant of endosymbiont-mediated inhibition of a wide range of pathogens, including RNA viruses, such as *Togaviridae*.

A. mellifera has a well characterized age-related division of labor (DOL), where, initially, newborn bees assume nursing functions and change them during their lifetime until they become foragers at 3 weeks old (Greenberg et al., 2012). This DOL provides an excellent condition to study the role of genes in natural behavioral plasticity. Based on that, Behura & Whitfield (2010) observed and compared patterns of gene expressions in the brains of honeybees: young nurse, young forager, old nurse, and old forager. The results showed that specific miRNA genes, including *miR-283*, are coordinately up-regulated in the late stages of honeybee life because of the high expression in the old forager bee in relation to other groups. Implying that behavioral maturation, switching from nursing to foraging activity, is influenced by regulatory pathways.

In another study involving cadmium tolerance in *Daphnia pulex,* S. Chen et al (2016) predicted that *miR-283* targets Rho guanine nucleotide exchange factor 10 (ARHGEF10). Inferring that *miR-283* family is involved in the GTPase regulatory network. GTPase is responsible for regulating body size in *Drosophila* (Kaplan et al., 2008)*,* and the individuals that exhibit low levels of GTPase, although they present a normal body proportion, have smaller body sizes. Therefore, as a result of the toxicity of cadmium, the progeny (F1, F2, and F3) of the individuals that were exposed to the metal showed an up-regulated *miR-283*, and smaller body sizes, by down-regulating the GTPase pathway.

3.3.4. miR-927

Nineteen miRNA precursors for *miR-927* family were identified, and thirty-seven mature miRNAs were distributed in the eight species. The miRNAs from bees of this family showed high conservation in relation to their orthologs, both for the analysis of primary and secondary structures (Fig. 17 and 18).

The tree generated from the phylogenetic analysis of the *miR-927* family showed a distribution in three well-defined clades (Diptera, Lepidoptera, and Hymenoptera). The Hymenoptera clade was composed of superfamilies: Pteromalidae, Vespidae, Apidae, and Halictidae. In addition, there was a correct grouping between all precursor sequences of this family with their respective orthologous species. This distribution showed similarity with the tree of life, where bees remained alongside other Hymenoptors (Fig. 19).

In other studies, *miR-927* (*ame-mir-927a*) was also identified in the bee *A. mellifera*, corroborating the sequences identified in this study (Greenberg et al., 2012; MacEdo et al., 2016; Weaver et al., 2007). The *miR-927* family sequences were found in several organisms belonging to the Superorder Endopterygota, including the organisms: *Drosophila virilis* (Ninova et al., 2014), *Bactrocera dorsalis* (Calla & Geib, 2015), *Polistes canadenses* and *Dinoponera quadriceps* (Patalano et al., 2015). In other studies, *miR-927* was also identified in mosquito vectors: *Aedes albopictus*, *Aedes aegypti,* and *Culex quinquefasciatus* (S. Li et al., 2009; Skalsky et al., 2010).

The cellular processes with which *miR-927* is related are gradually being elucidated. He et al (2020) demonstrated the property of *miR-927* in the regulation of development and metamorphosis of insects in *D. melanogaster*, since these miRNAs decreased the expression of the Kruppel homolog1 gene (Kr-h1); The authors further reported that *miR-927* expression was repressed by JH thus demonstrating that miRNA mechanisms that regulate insect development and metamorphosis via Kr-h1 targeting are conserved and helping to understand the interaction between JH / miRNAs / Kr-h1.

JH's mechanism of action is to inhibit metamorphosis in insects until the larvae reach an appropriate size, and its production decreases with the simultaneous increase in the body and morphological changes that are completed from the final stage of pupa to adult (Belles, 2019). The JH primary response gene, Kr-h1, is an important ant metamorphic factor in several species of hemimetabolous and holometabolous insects. The transcriptional regulation of KR-H1 by JH is via direct binding of the JH receptor, MET/GCE, and the JH response element (Ebox or E-box-like motif) within the promoter of Kr-h1. Therefore, if JH is required for larval maintenance, the same is true for Kr-h1 (Kayukawa et al., 2014).

Given that the Kr-h1 protein is relatively well conserved among *Apis mellifera* bee species (Fussnecker & Grozinger, 2008; Grozinger et al., 2003) and the property of the interaction between JH and miR-927 in the regulation of insect development has been demonstrated by *miR-927* decreasing the expression of Kr-h1, there the need for further studies to determine the exact molecular interaction JH/miR-927/Kr-h1 in bees.

Avila-Bonilla et al (2020) described the ability of *miR-927* to modify the expression of effector genes in the immune response in mosquitoes. While studying *A. Aegypti* mosquito cells infected by Dengue Virus serotype 2 (DENV-2), they assessed the participation of miRNAs during infection and observed that among the modulated miRNAs, *miR-927* showed the highest overexpression, having an important role during the replicative cycle of DENV-2. The authors proposed the ability of *miR-927* to target the 3' UTR end of the Filamin gene and regulate its expression. Filamins are essential in rearranging the cytoskeletal protein Actin, which is necessary for the proper function of several membrane receptors. Thus, it suggests

that *miR-927* expression can regulate the innate immune response in mosquitoes through Filamin repression to promote DENV infection. The participation of *miR-927* in the innate antiviral immune response and other immunological developments remains to be elucidated in bees (Avila-Bonilla et al., 2020).

3.4. miRNA target genes of in *A. mellifera*

After the maturation pathway, miRNA is ready to act in mRNA to suppress expression. The mechanism behind this is the complementarity of 3' UTR from mRNA and the seed region of miRNA. This information was a conclusion from an important work by Lee *et al.* (1993), where they identified transcripts from *lin-4* were complementary to 3' UTR from *lin-14* mRNA. The seed region is from the nucleotides 2 through 8, the complementarity is the type of Watson-Crick, and it is distinguished by the perfect match, with no gaps in the structure miRNA: mRNA alignment (Peterson et al., 2014).

Aside from the alignment between the sequences, some other features are considered in miRNA target prediction. In our case, *miRanda* and *RNAhybrid* tools were used. m*iRanda* measures the thermodynamic free energy (Gibbs free energy) properties and the conservation level in the species analyzed, meanwhile *RNAhybrid* calculates the better option energetically based on miRNA hybridization (John et al., 2004; Rehmsmeier et al., 2004; Xiao et al., 2009).

So, considering the particularities described above, Supplementary data VII - Table I, shows us all the predicted targets for each miRNA, with the total number of potential targets predicted per miRNA in *A. mellifera*. We made a global target prediction regarding all mature miRNAs against the 3' UTR data contained in the GFF file. It is important to imply the importance of this data obtained and to aim to objectify our research, and we consider the importance of the protein Vitellogenin (Vg) in a range of biological processes in bees and insects as a whole. Vg, known as yolk protein, is a glycoprotein with approximately 180 kDa and is widely studied in honeybees (Seehuus et al., 2006). VG is synthesized in fat tissues, reaching the oocytes by endocytosis through specific receptors (Agostini et al., 2021). Vg suffers many chemical alterations such as glycosylation and phosphorylation (Raikhel & Dhadialla, 1992). Vg is considered the most important protein in oocyte maturation in insects, making it crucial in oviparous reproduction, mainly because of its involvement in embryonic development (Carducci et al., 2019; Wu et al., 2021). In *A. mellifera,* Vg is considered critical for queens, as they are involved in egg maturation and exhibit antioxidant properties, contributing to their longer life-spans (Corona et al., 2007; Ihle et al., 2015). A peculiarity about Vg is that since it is connected to fertility, it was expected only queens to be responsible for its production, but workers also show Vg levels. This occurs because Vg is related to pathogen recognition receptors and helps with trans-generational immunity and gustatory perceptions (Salmela et al., 2015; Wang et al., 2012; S. Zhang et al., 2015).

In social bees, the organization and division of labor are important traits in workers. Dictated by age, newborn bees assume nursing functions and change them during their lifetime, becoming foragers at three weeks old (Robinson, 1997). Recently, Vg levels were correlated with age evolution in workers, with higher titers being found in nurses and decreasing throughout life (Nelson et al., 2007).

miRNAs were searched with probable action in Vg control. Seven potential miRNAs were related with Vg control, *ame-mir-1b-5p, ame-mir-283-5p, ame-mir-3774-3p, ame-mir-467g-3p, ame-mir-8460-3p, ame-mir-927-5p, and ame-mir-9896-5p.* The involvement of miRNAs in Vg controlling has already been

thoroughly studied. This control is also mediated by JH, 20-Hydroxyecdysone (20E), and nutritional conditions, but mostly by miRNAs, which also affect the previous mediators (Wu et al., 2021). Zhang demonstrated that *miR-989* in *A. aegypti* has Vg as one of their targets (X. Zhang et al., 2017), and in *A. mellifera* this kind of study is being proposed for the very first time, which means we are presenting unprecedented results.

4. Conclusions

Considering the global importance of bees, especially as pollinators agents, only the worldwide population rate and the need for a better understanding of the particularities of the interaction and molecular regulation of their physiology and metabolism in order to better point out strategies for the production and conservation of these species, our results expand the study of miRNAs in bee by providing a better understanding of their essential roles in the miRNA-based regulation processes in bee, their processing pathways, and gene expression, as well as providing targets for future investigations. The results elucidated several aspects of miRNAs in these eight species.

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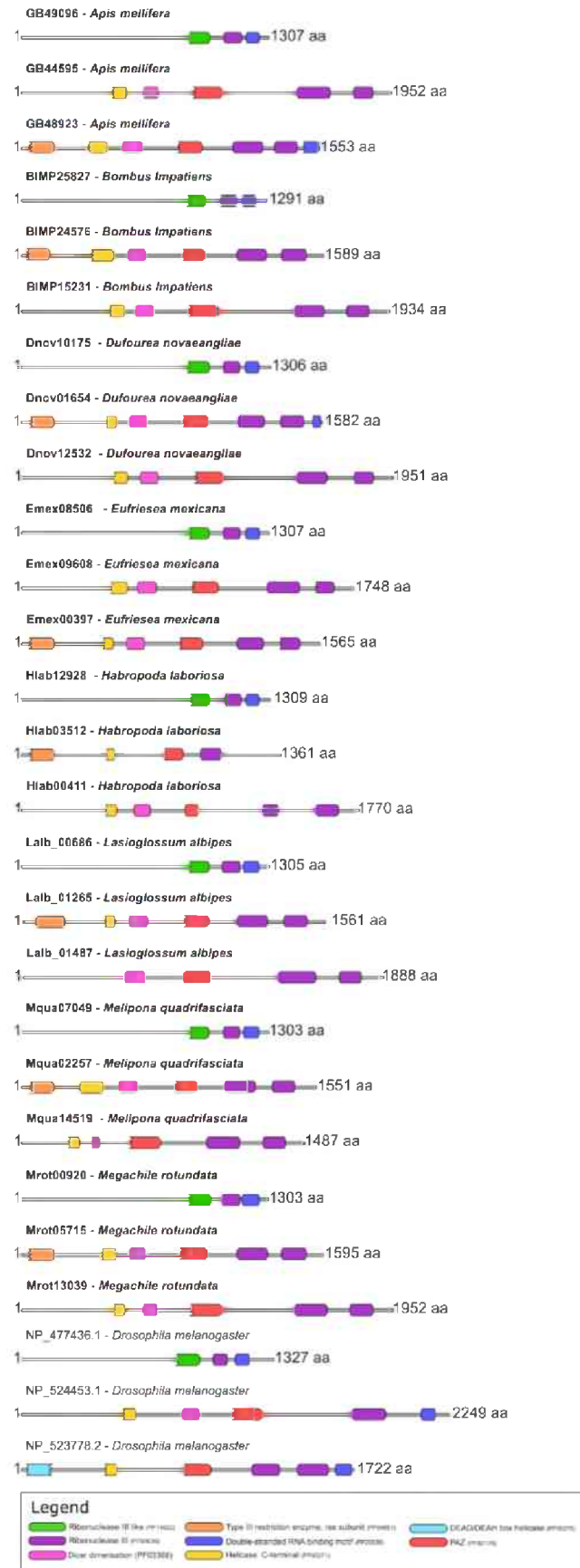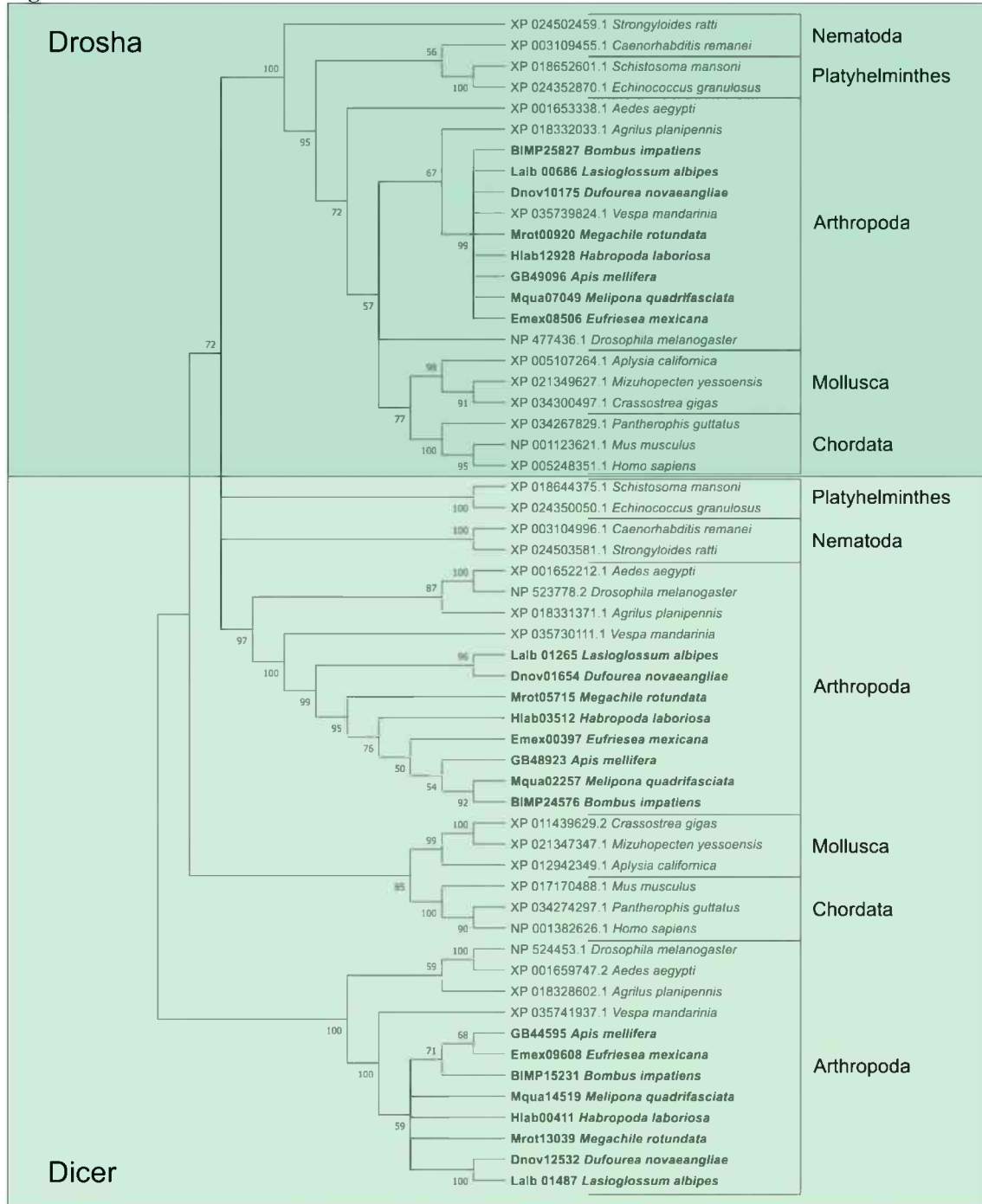
Fig. 1

Fig. 2
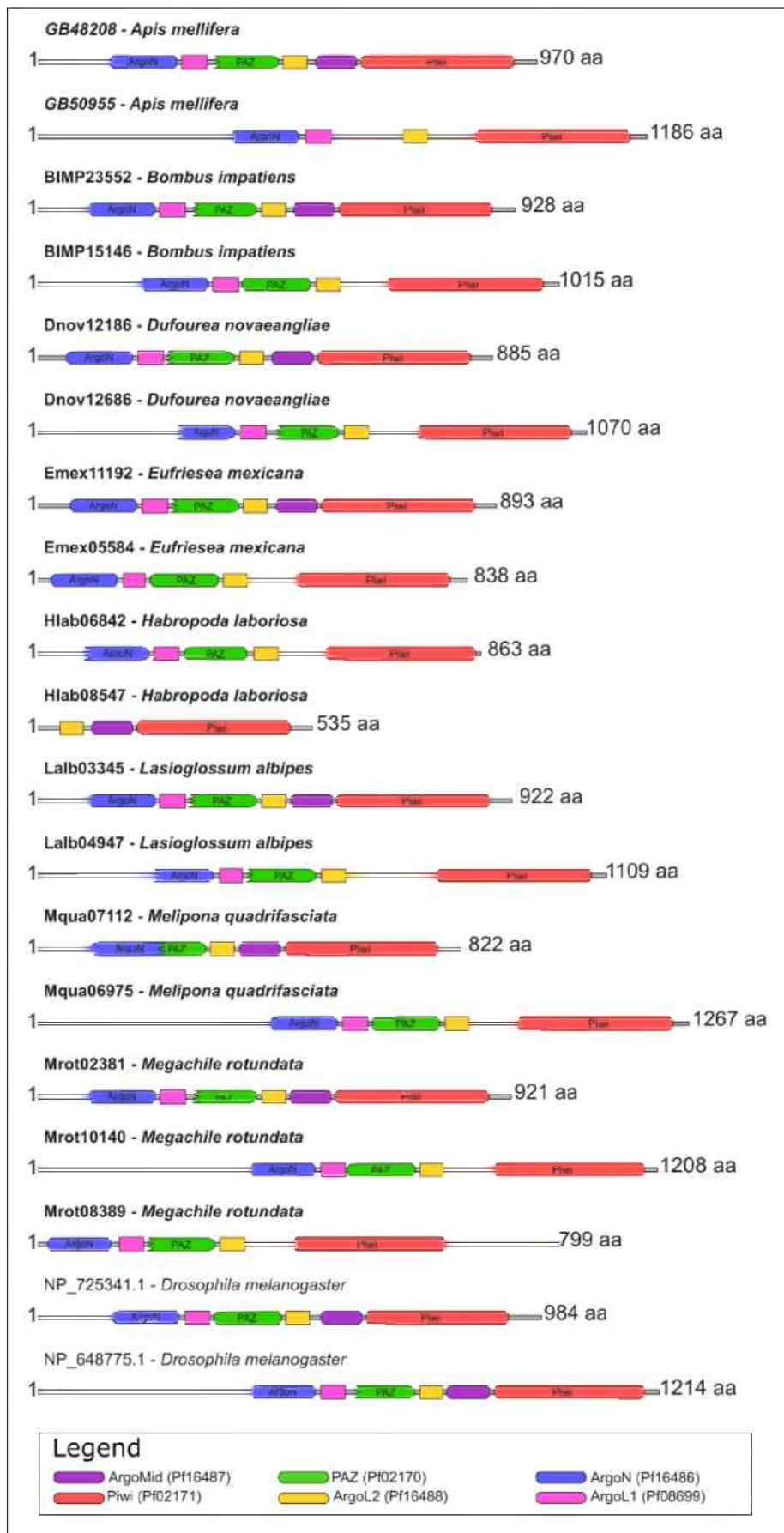
Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

A

B

C

Fig.8

Fig. 9

ame-miR-1b   bim-miR-1b   dno-miR-1b   eme-miR-1b   hla-miR-1b

lal-miR-1b   mqu-miR-1b   mro-miR-1b   dqu-miR-1b   mse-miR-1b

Legend:

0 ▬▬▬▬▬▬ 1

Base-pair probabilities

Fig. 10

Fig. 11

Apis mellifera *Bombus impatiens* *Dufourea novaeangliae* *Eufriesea mexicana*

Habropoda laboriosa *Lasioglossum albipes* *Melipona quadrifasciata* *Megachile rotundata*
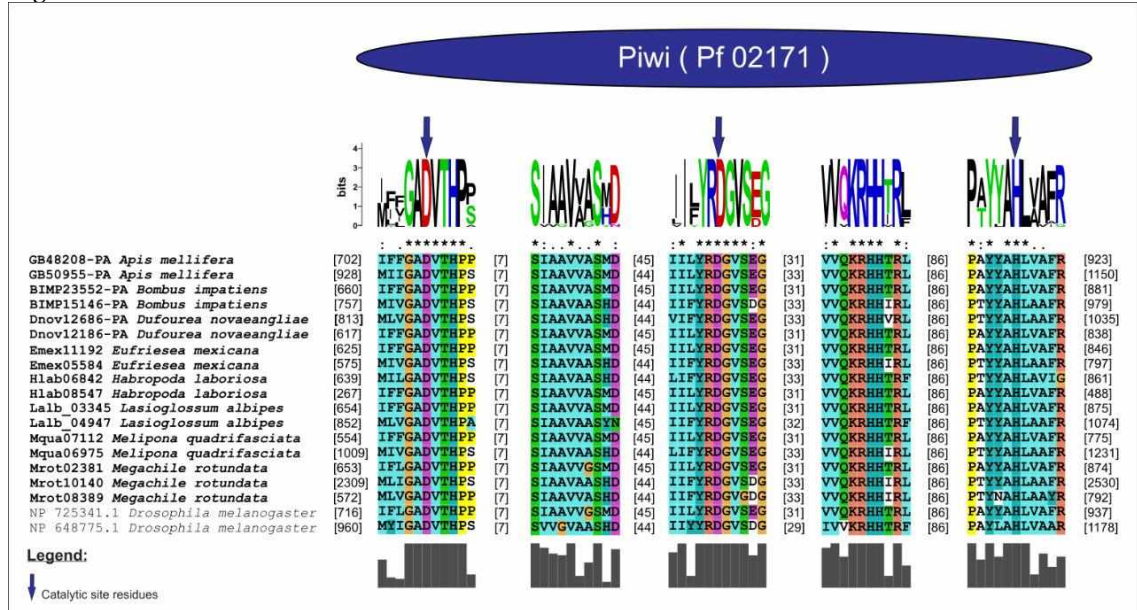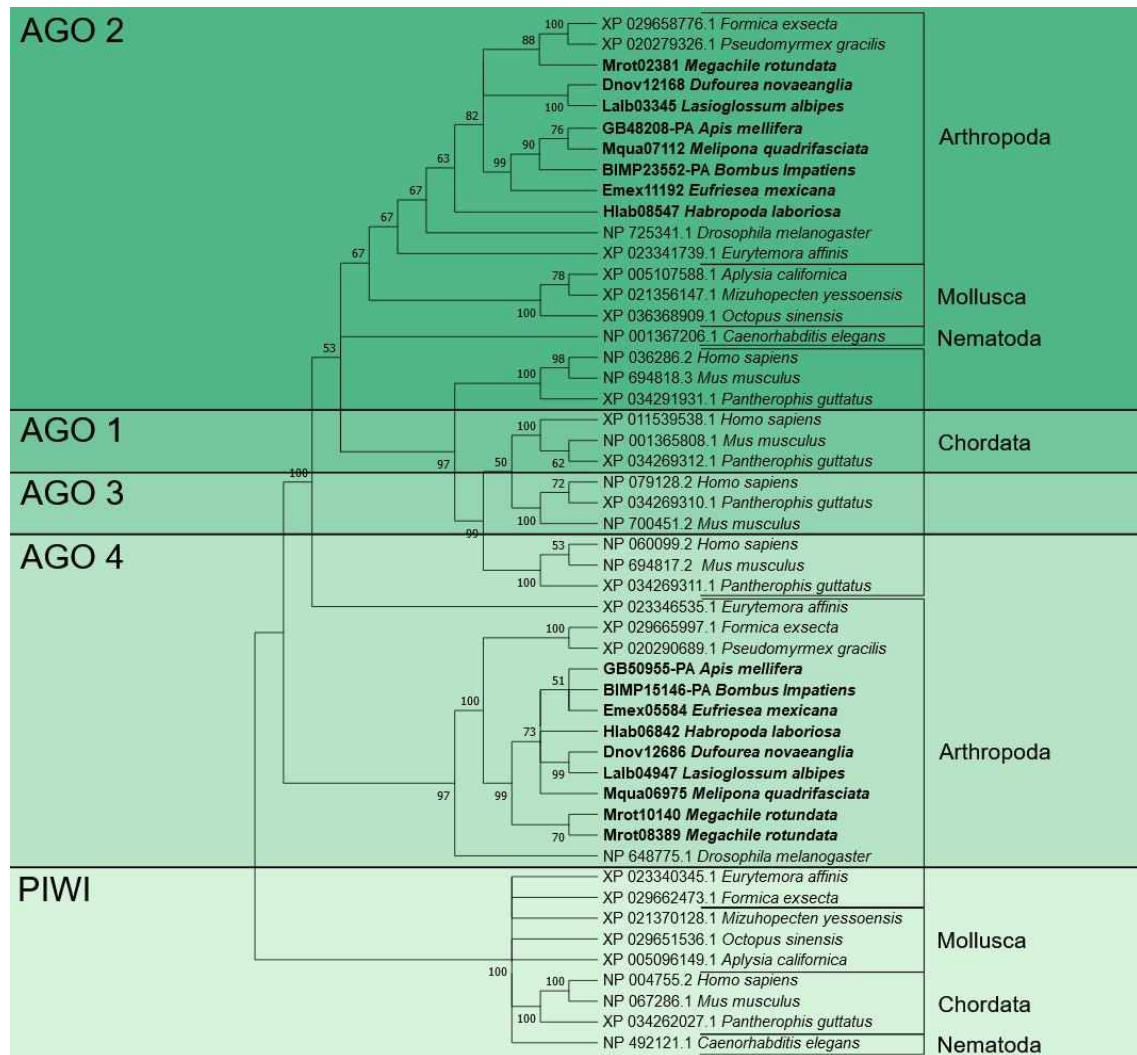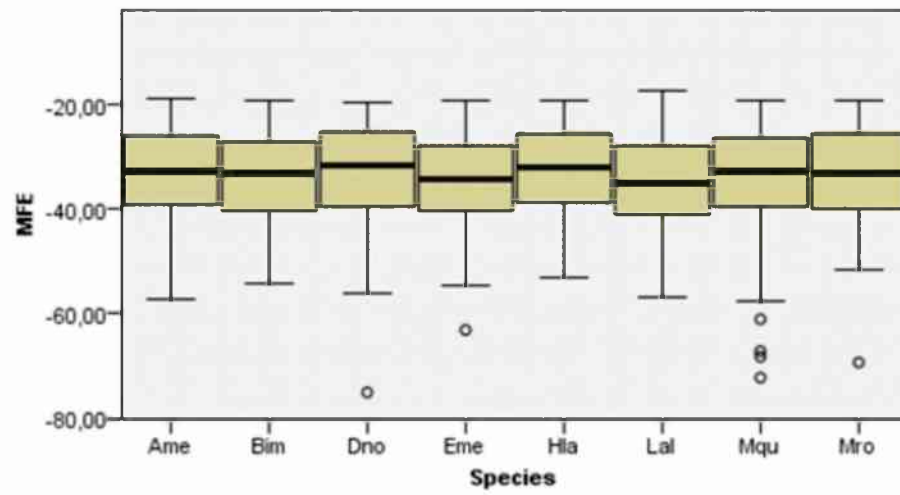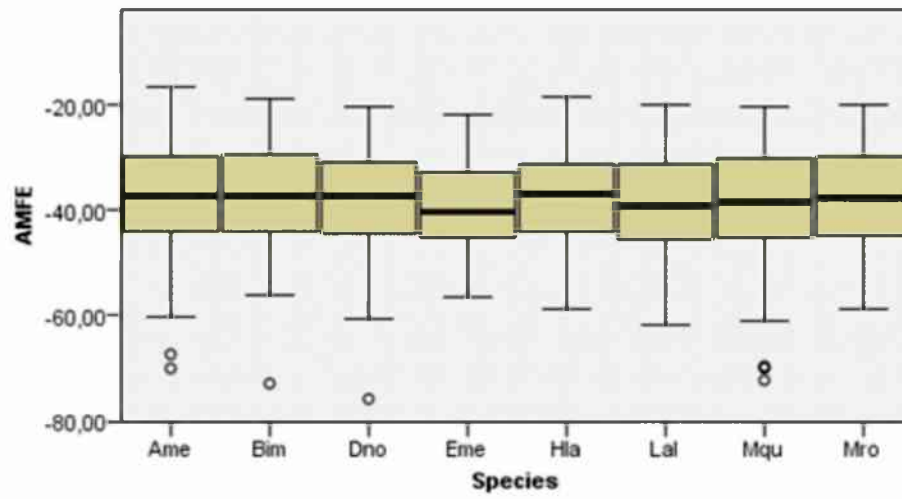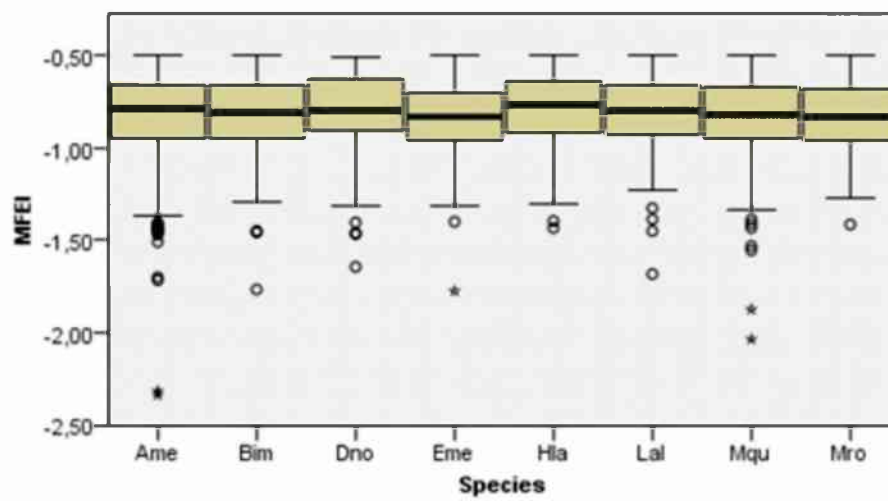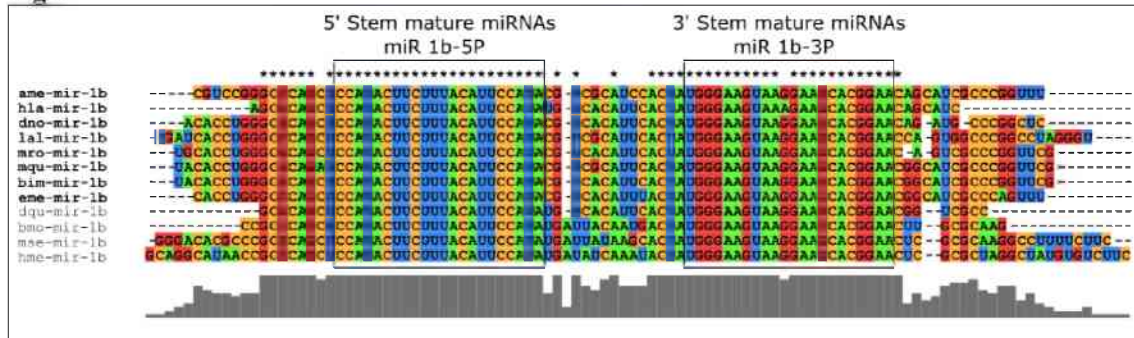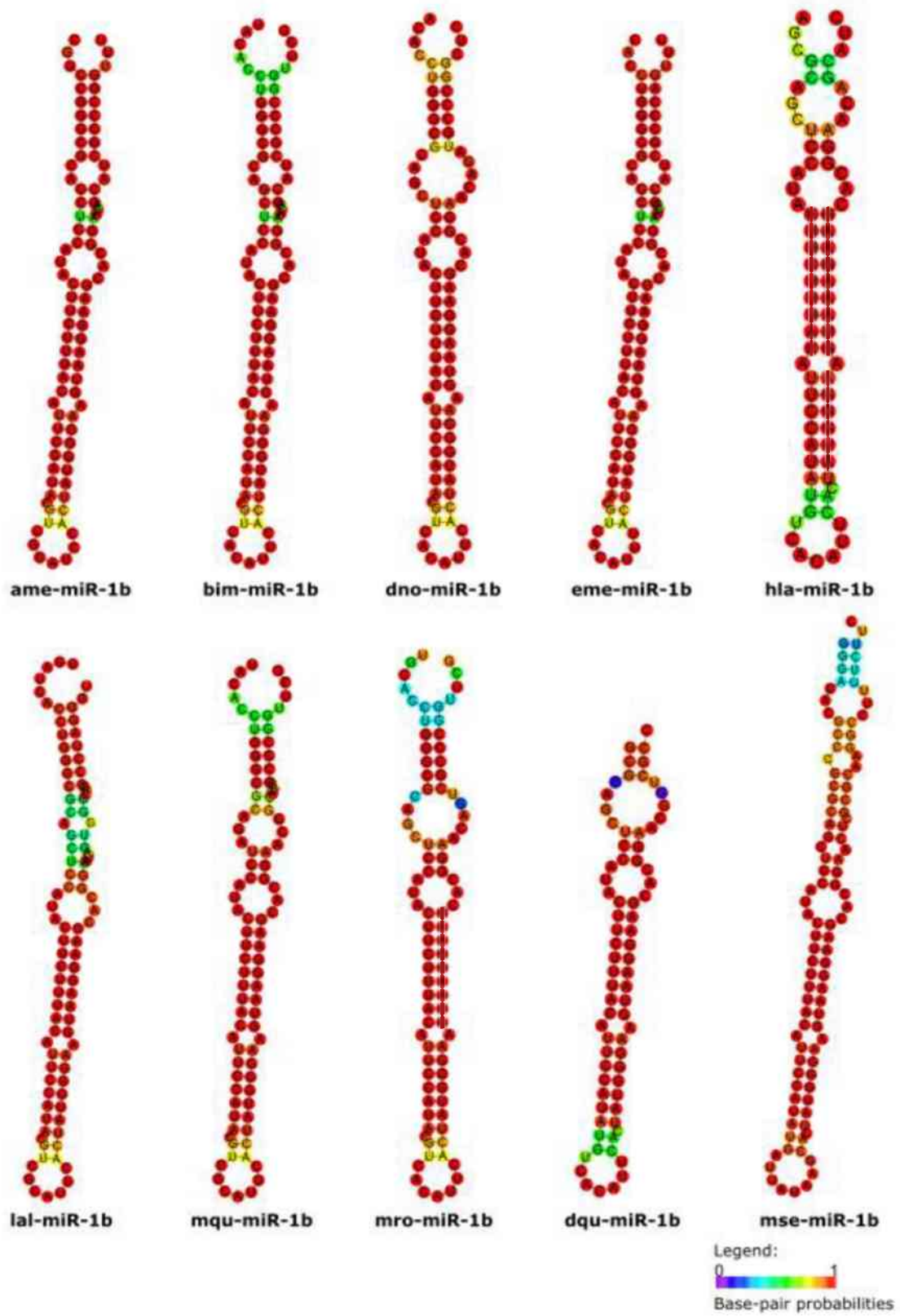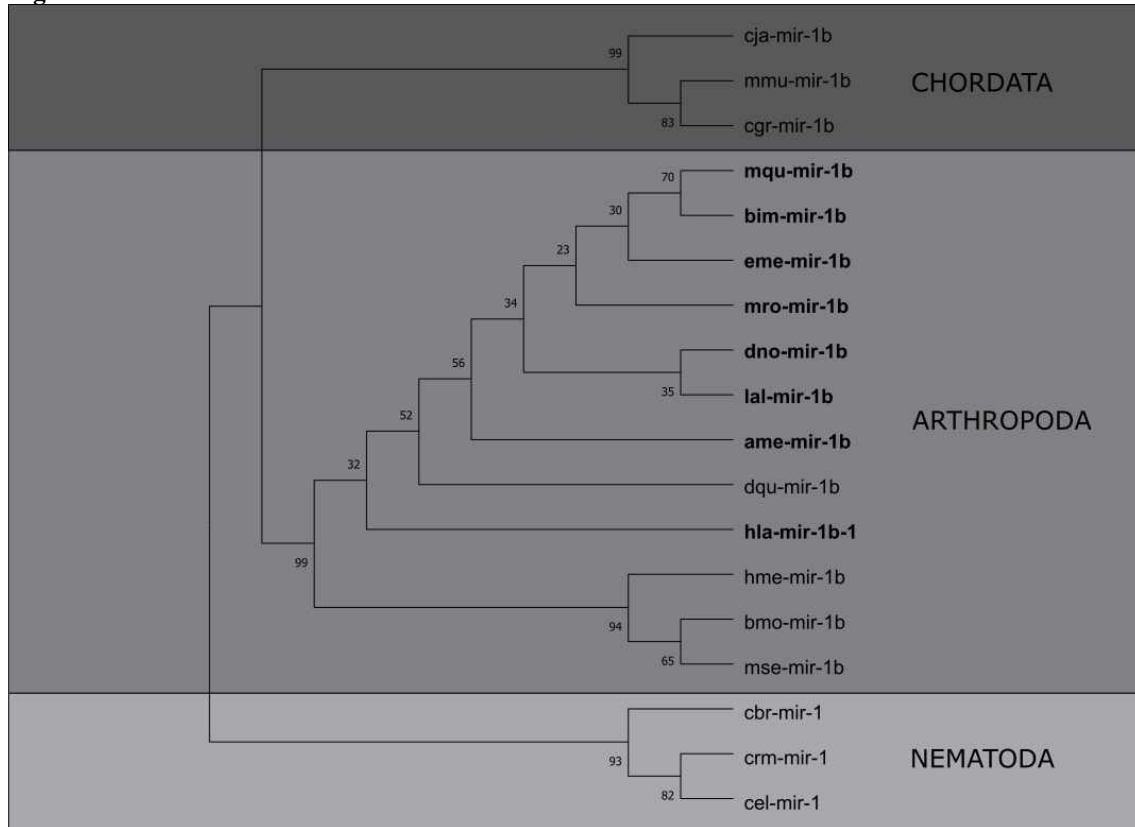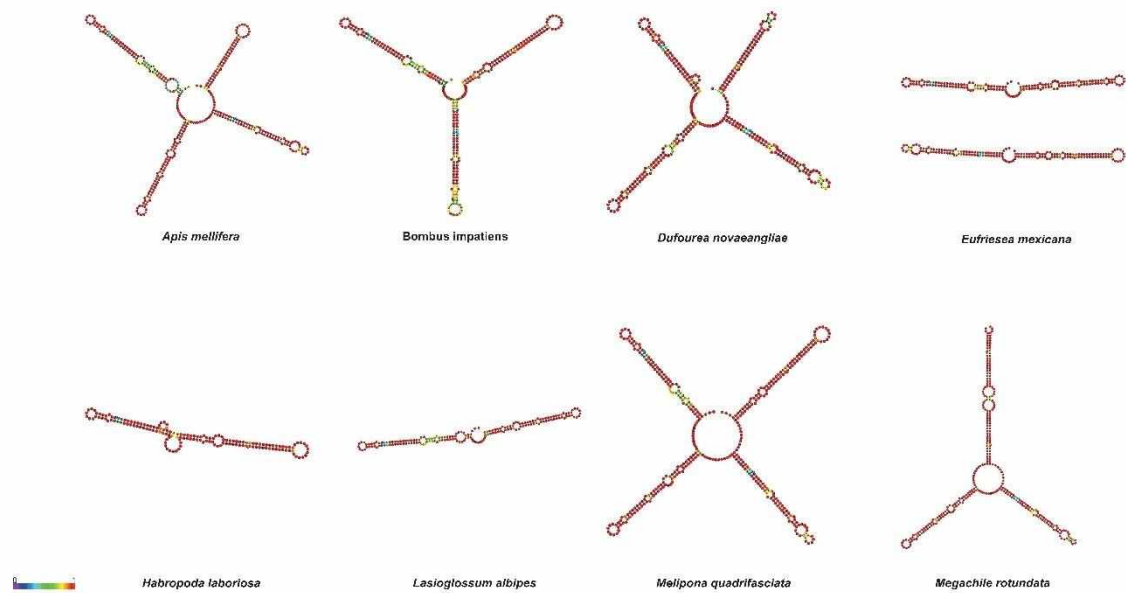
Fig. 12 – A, B and C
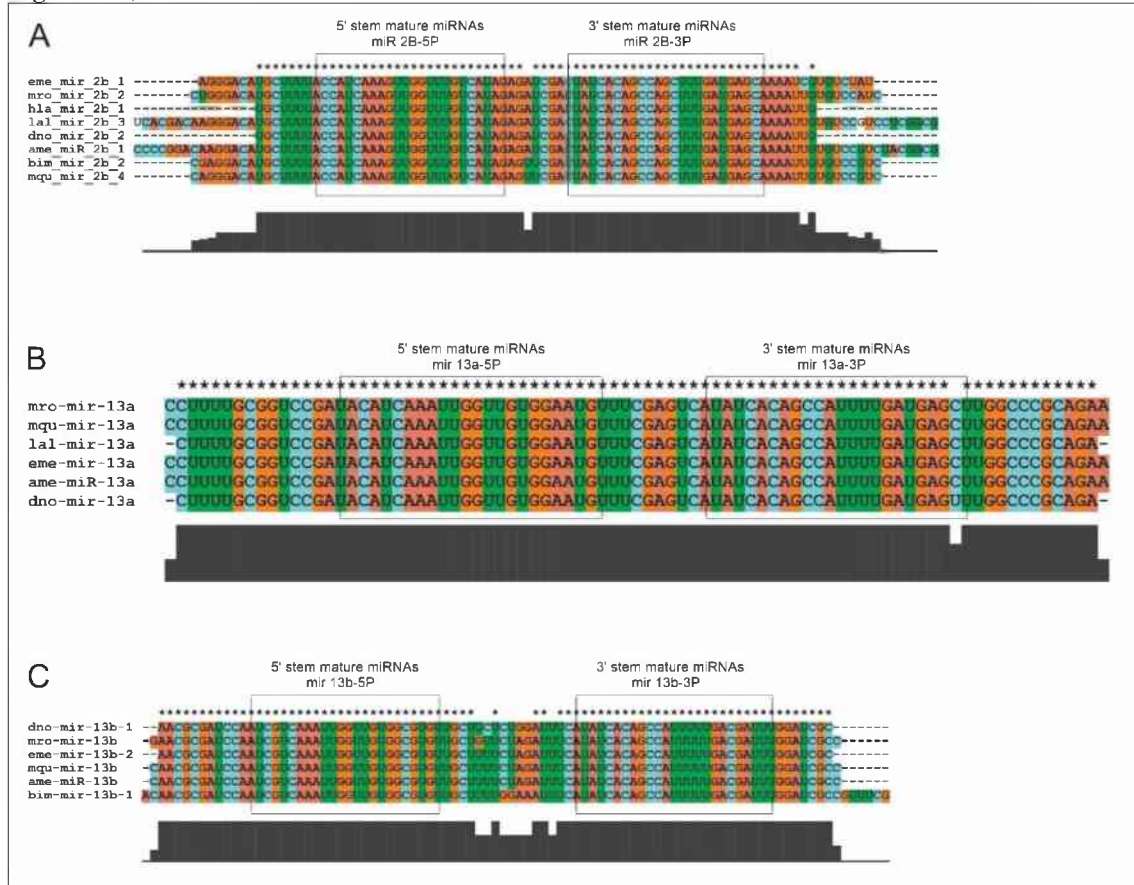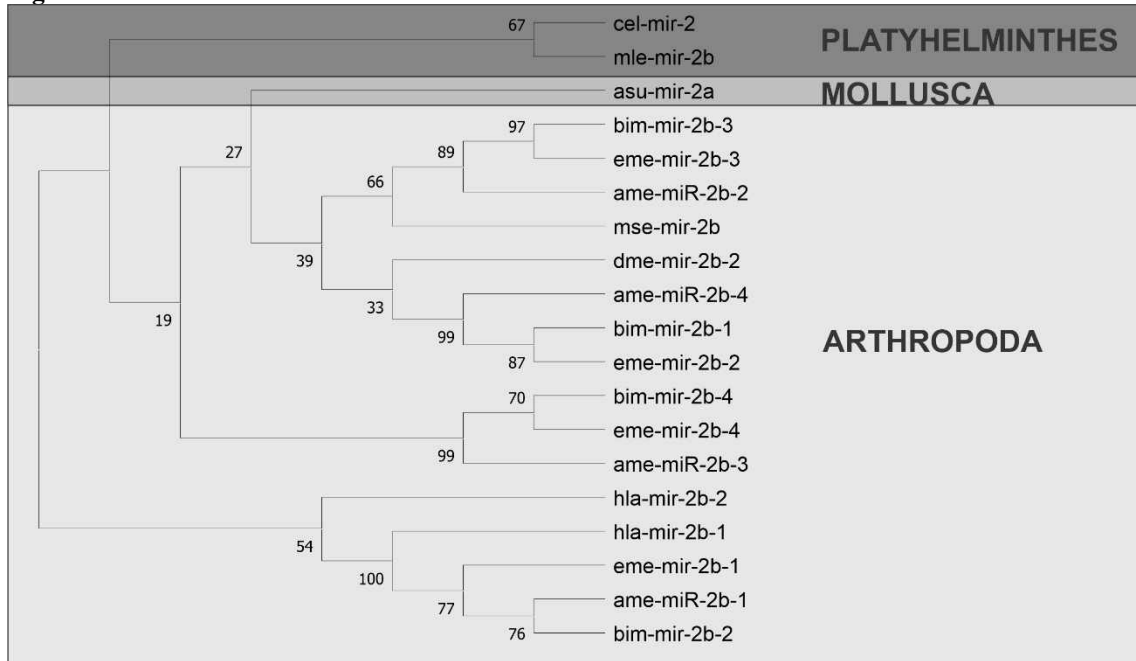
Fig. 13A

Fig. 13B
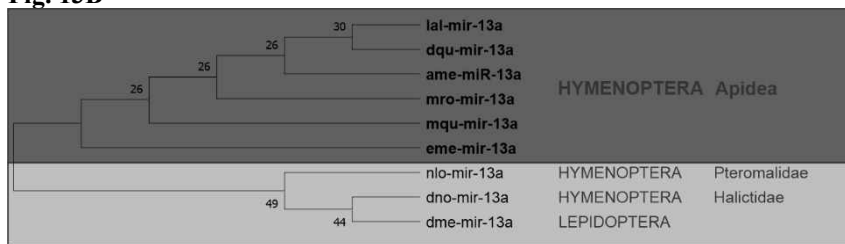
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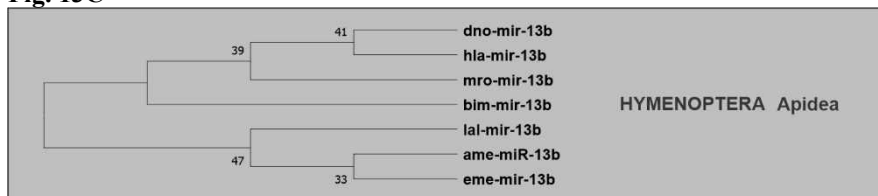
Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

ame-miR-927 bim-miR-927 hla-miR-927 eme-miR-927 dno-miR-927 lal-miR-927 mqu-miR-927 mqu-miR-927

Fig. 19

tca-mir-927a	COLEOPTERA	
aga-mir-927		
dsi-mir-927	DIPTERA	
dme-mir-927		
dps-mir-927		
dvi-mir-927		
bdo-mir-927		
bmo-mir-927	LEPIDOPTERA	
hme-mir-927		
api-mir-927	HEMIPTERA	
ngi-mir-927	HYMENOPTERA	Pteromalidae
nlo-mir-927		
nvi-mir-927		
pca-mir-927a	HYMENOPTERA	Vespidae
eme-mir-927	HYMENOPTERA	Apidea
dno-mir-927	HYMENOPTERA	Halictidae
bim-mir-927	HYMENOPTERA	Apidea
mqu-mir-927		
hla-mir-927		
lal-mir-927		
ame-miR-927		

SUPPLEMENTARY DATA

Table S1: List of proteins involved in miRNA pathway and localized, by homology strategy, in the eight species of bee in subject. GB: *Apis mellifera*; BIMP: *Bombus impatiens*; Dnov: *Dufourea novaeangliae*; Emex: *Eufriesea mexicana*; Hlab: *Habropoda laboriosa*; Lalb: *Lasioglossum albipes*; Mqua: *Melipona quadrifasciata*; Mrot: *Megachile rotundata.*

Tables S2: Representation of domains composition and localization of putative proteins: Table S2.1 Drosha, S2.2 Dicer and S2.3 Argonaute.

Tables S3: Final table generated by algorithm described on the material and methods. This material brings all the miRNAs located in the whole genome of *A. mellifera* (S3.1), *B. impatiens* (S3.2), *D. novaeangliae* (S3.3), *E. mexicana* (S3.4), *H. l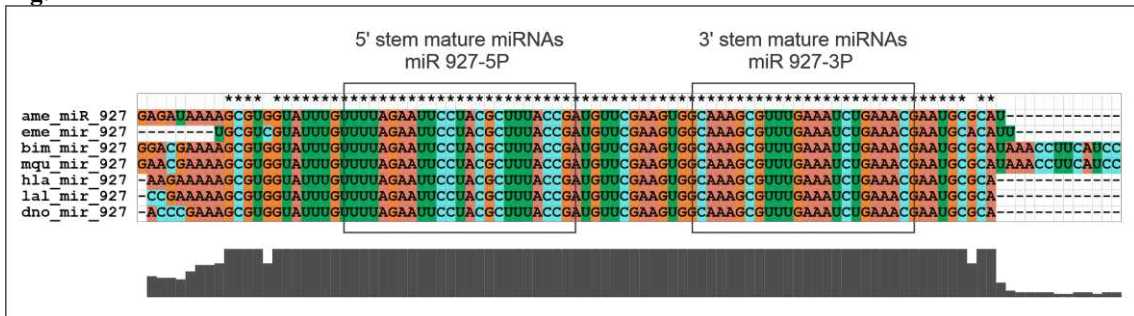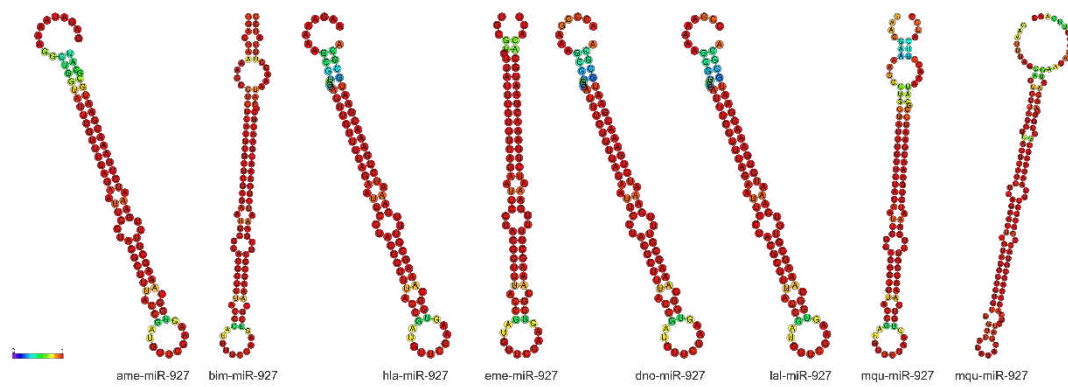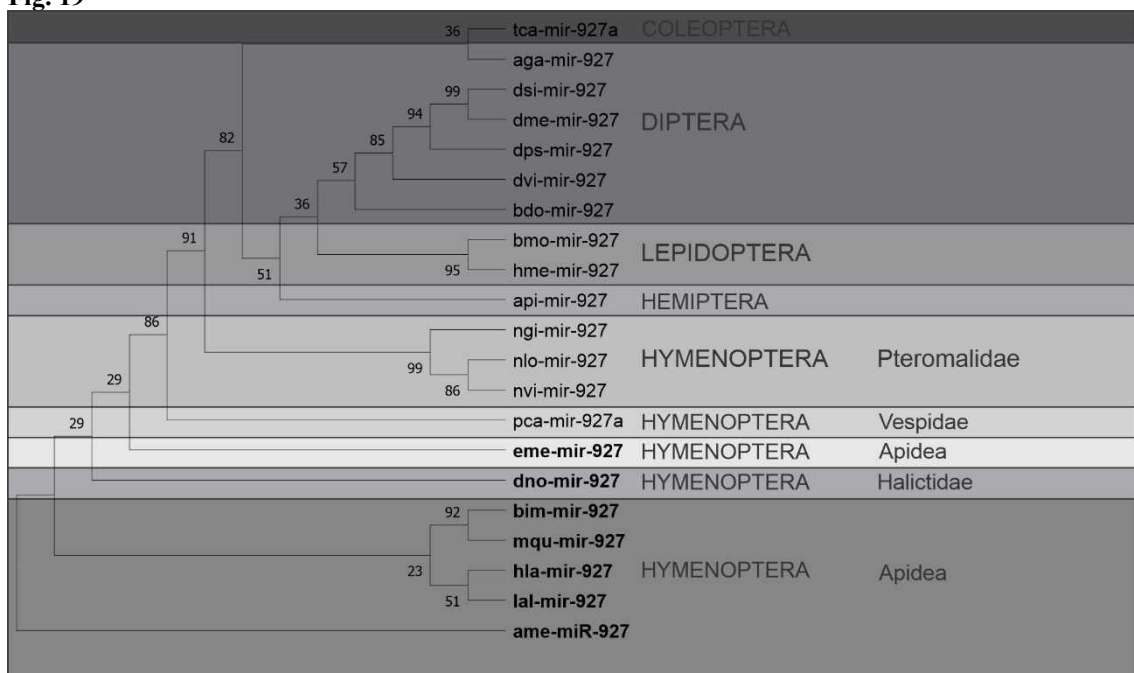aboriosa* (S3.5), *L. albipes* (S3.6), *M. rotundata* (S3.7), *M. quadrifasciata* (S3.8). All tables show both precursor and mature miRNAs, as well as the position of mature within the precursor.

Tables S4: Demonstration of the number mature miRNAs, by families, founded in each specie (S4.1), and demonstration of the most prevalent miRNAs families in the correlated species (S4.2).

Tables S5: Table containing the data about MFE, AMFE and MFEI of miRNAs in all eight related species *A. mellifera* (S5.1), *B. impatiens* (S5.2), *D. novaeangliae* (S5.3), *E. mexicana* (S5.4), *H. laboriosa* (S5.5), *L. albipes* (S5.6), *M. rotundata* (S5.7), *M. quadrifasciata* (S5.8).

Table S6: Genomic localization of the miRNA family miR-2b (mir-2b, mir-13a and b, and mir-71) demonstrating the possibility of cluster formation.

Table S7: Number of presumable targets for each miRNA family.

ANEXOS:

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Instructions for Authors

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Online Submission

Please follow the hyperlink "Submit manuscript" and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)

- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country

- A clear indication and an active e-mail address of the corresponding author

- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials

- Trial registration number and date of registration, followed by "retrospectively registered", for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

Competing Interests: Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to "Competing Interests and Funding" below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).

- This result was later contradicted by Becker and Seligman (1996).

- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

Reference list entries should be alphabetized by the last names of the first author of each work. Please alphabetize according to the following rules: 1) For one author, by name of author, then chronologically; 2) For two authors, by name of author, then name of coauthor, then chronologically; 3) For more than two authors, by name of first author, then chronologically.

If available, please always include DOIs as full DOI links in your reference list (e.g. "https://doi.org/abc").

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN LTWA

If you are unsure, please use the full journal title.

Tables

- All tables are to be numbered using Arabic numerals.

- Tables should always be cited in text in consecutive numerical order.

- For each table, please supply a table caption (title) explaining the components of the table.

- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.

- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

Electronic Figure Submission

- Supply all figures electronically.

- Indicate what graphics program was used to create the artwork.

- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.

- Vector graphics containing fonts must have the fonts embedded in the files.

- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

- Definition: Black and white graphic with no shading.

- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

- All lines should be at least 0.1 mm (0.3 pt) wide.

- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

- Vector graphics containing fonts must have the fonts embedded in the files.

- Definition: Photographs, drawings, or paintings with fine shading, etc.

- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

- Halftones should have a minimum resolution of 300 dpi.

- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.

- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).

- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.

- Avoid effects such as shading, outline letters, etc.

- Do not include titles or captions within your illustrations.

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- All figures are to be numbered using Arabic numerals.

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- Figure parts should be denoted by lowercase letters (a, b, c, etc.).

- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.

- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.

- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- Figures should be submitted within the body of the text. Only if the file size of the manuscript causes problems in uploading it, the large figures should be submitted separately from the text.

- When preparing your figures, size figures to fit in the column width.

- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.

- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

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Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)

- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)

- Any figure lettering has a contrast ratio of at least 4.5:1

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Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal's Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.

- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.

- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

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- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.

- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

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Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

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- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.

- Refer to the supplementary files as "Online Resource", e.g., "... as shown in the animation (Online Resource 3)", "... additional data are given in Online Resource 4".

- Name the files consecutively, e.g. "ESM_3.mpg", "ESM_4.pdf".

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Accessibility

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- The manuscript contains a descriptive caption for each supplementary material

- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

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This journal is committed to upholding the integrity of the scientific record. As a member of the Committee on Publication Ethics (COPE) the journal will follow the COPE guidelines on how to deal with potential acts of misconduct.

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- A single study should not be split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (i.e. 'salami-slicing/publishing').

- Concurrent or secondary publication is sometimes justifiable, provided certain conditions are met. Examples include: translations or a manuscript that is intended for a different group of readers.

- Results should be presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation). Authors should adhere to discipline-specific rules for acquiring, selecting and processing data.

- No data, text, or theories by others are presented as if they were the author's own ('plagiarism'). Proper acknowledgements to other works must be given (this includes material that is closely copied (near verbatim), summarized and/or paraphrased), quotation marks (to indicate words taken from another source) are

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Fundamental errors

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Compliance with Ethical Standards

To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, authors should include information regarding sources of funding, potential conflicts of interest (financial or non-financial), informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals.

Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest

- Research involving Human Participants and/or Animals

- Informed consent

Please note that standards could vary slightly per journal dependent on their peer review policies (i.e. single or double blind peer review) as well as per journal subject discipline. Before submitting your article check the instructions following this section carefully.

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

The Editors reserve the right to reject manuscripts that do not comply with the above-mentioned guidelines. The author will be held responsible for false statements or failure to fulfill the above-mentioned guidelines.

Competing Interests

Authors are requested to disclose interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work. Disclosure of interests provides a complete and transparent process and helps readers form their own judgments of potential bias. This is not meant to imply that a financial relationship with an organization that sponsored the research or compensation received for consultancy work is inappropriate.

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Interests that should be considered and disclosed but are not limited to the following:

Funding: Research grants from funding agencies (please give the research funder and the grant number) and/or research support (including salaries, equipment, supplies, reimbursement for attending symposia, and other expenses) by organizations that may gain or lose financially through publication of this manuscript.

Employment: Recent (while engaged in the research project), present or anticipated employment by any organization that may gain or lose financially through publication of this manuscript. This includes multiple affiliations (if applicable).

Financial interests: Stocks or shares in companies (including holdings of spouse and/or children) that may gain or lose financially through publication of this manuscript; consultation fees or other forms of remuneration from organizations that may gain or lose financially; patents or patent applications whose value may be affected by publication of this manuscript.

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Non-financial interests: In addition, authors are requested to disclose interests that go beyond financial interests that could impart bias on the work submitted for publication such as professional interests, personal relationships or personal beliefs (amongst others). Examples include, but are not limited to: position on editorial board, advisory board or board of directors or other type of management relationships; writing and/or consulting for educational purposes; expert witness; mentoring relations; and so forth.

Primary research articles require a disclosure statement. Review articles present an expert synthesis of evidence and may be treated as an authoritative work on a subject. Review articles therefore require a disclosure statement.Other article types such as editorials,

book reviews, comments (amongst others) may, dependent on their content, require a disclosure statement. If you are unclear whether your article type requires a disclosure statement, please contact the Editor-in-Chief.

Please note that, in addition to the above requirements, funding information (given that funding is a potential competing interest (as mentioned above)) needs to be disclosed upon submission of the manuscript in the peer review system. This information will automatically be added to the Record of CrossMark, however it is not added to the manuscript itself. Under 'summary of requirements' (see below) funding information should be included in the 'Declarations' section.

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Funding' and/or 'Competing interests'. Other declarations include Ethics approval, Consent, Data, Material and/or Code availability and Authors' contribution statements.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

When all authors have the same (or no) conflicts and/or funding it is sufficient to use one blanket statement.

Examples of statements to be used when funding has been received:

- Partial financial support was received from [...]

- The research leading to these results received funding from […] under Grant Agreement No[…].

- This study was funded by […]

- This work was supported by […] (Grant numbers […] and […]

Examples of statements to be used when there is no funding:

- The authors did not receive support from any organization for the submitted work.

- No funding was received to assist with the preparation of this manuscript.

- No funding was received for conducting this study.

- No funds, grants, or other support was received.

Examples of statements to be used when there are interests to declare:

- Financial interests: Author A has received research support from Company A. Author B has received a speaker honorarium from Company Wand owns stock in Company X. Author C is consultant to company Y.

  Non-financial interests: Author C is an unpaid member of committee Z.

- Financial interests: The authors declare they have no financial interests.

  Non-financial interests: Author A is on the board of directors of Y and receives no compensation as member of the board of directors.

- Financial interests: Author A received a speaking fee from Y for Z. Author B receives a salary from association X. X where s/he is the Executive Director.

  Non-financial interests: none.

- Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M and Company N. Dr. C has received speaker honorarium and research funding from Company M and Company O. Author D has received travel support from Company O.

  Non-financial interests: Author D has served on advisory boards for Company M, Company N and Company O.

Examples of statements to be used when authors have nothing to declare:

- The authors have no relevant financial or non-financial interests to disclose.

- The authors have no competing interests to declare that are relevant to the content of this article.

- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

- The authors have no financial or proprietary interests in any material discussed in this article.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research involving human participants, their data or biological material

Ethics approval

When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

Retrospective ethics approval

If a study has not been granted ethics committee approval prior to commencing, retrospective ethics approval usually cannot be obtained and it may not be possible to consider the manuscript for peer review. The decision on whether to proceed to peer review in such cases is at the Editor's discretion.

Ethics approval for retrospective studies

Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain) ethics approval may be required dependent on the law and the national ethical guidelines of a country. Authors should check with their institution to make sure they are complying with the specific requirements of their country.

Ethics approval for case studies

Case reports require ethics approval. Most institutions will have specific policies on this subject. Authors should check with their institution to make sure they are complying with the specific requirements of their institution and seek ethics approval where needed. Authors should be aware to secure informed consent from the individual (or parent or guardian if the participant is a minor or incapable) See also section on Informed Consent.

Cell lines

If human cells are used, authors must declare in the manuscript: what cell lines were used by describing the source of the cell line, including when and from where it was obtained,

whether the cell line has recently been authenticated and by what method. If cells were bought from a life science company the following need to be given in the manuscript: name of company (that provided the cells), cell type, number of cell line, and batch of cells.

It is recommended that authors check the NCBI database for misidentification and contamination of human cell lines. This step will alert authors to possible problems with the cell line and may save considerable time and effort.

Further information is available from the International Cell Line Authentication Committee (ICLAC).

Authors should include a statement that confirms that an institutional or independent ethics committee (including the name of the ethics committee) approved the study and that informed consent was obtained from the donor or next of kin.

Research Resource Identifiers (RRID)

Research Resource Identifiers (RRID) are persistent unique identifiers (effectively similar to a DOI) for research resources. This journal encourages authors to adopt RRIDs when reporting key biological resources (antibodies, cell lines, model organisms and tools) in their manuscripts.

Examples:

Organism: Filip1tm1a(KOMP)Wtsi RRID:MMRRC_055641-UCD

Cell Line: RST307 cell line RRID:CVCL_C321

Antibody: Luciferase antibody DSHB Cat# LUC-3, RRID:AB_2722109

Plasmid: mRuby3 plasmid RRID:Addgene_104005

Software: ImageJ Version 1.2.4 RRID:SCR_003070

RRIDs are provided by the Resource Identification Portal. Many commonly used research resources already have designated RRIDs. The portal also provides authors links so that they can quickly register a new resource and obtain an RRID.

Clinical Trial Registration

The World Health Organization (WHO) definition of a clinical trial is "any research study that prospectively assigns human participants or groups of humans to one or more health-

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To ensure the integrity of the reporting of patient-centered trials, authors must register prospective clinical trials (phase II to IV trials) in suitable publicly available repositories. For example www.clinicaltrials.gov or any of the primary registries that participate in the WHO International Clinical Trials Registry Platform.

The trial registration number (TRN) and date of registration should be included as the last line of the manuscript abstract.

For clinical trials that have not been registered prospectively, authors are encouraged to register retrospectively to ensure the complete publication of all results. The trial registration number (TRN), date of registration and the words 'retrospectively registered' should be included as the last line of the manuscript abstract.

Standards of reporting

Springer Nature advocates complete and transparent reporting of biomedical and biological research and research with biological applications. Authors are recommended to adhere to the minimum reporting guidelines hosted by the EQUATOR Network when preparing their manuscript.

Exact requirements may vary depending on the journal; please refer to the journal's Instructions for Authors.

Checklists are available for a number of study designs, including:

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Observational studies (STROBE)

Systematic reviews and meta-analyses (PRISMA) and protocols (Prisma-P)

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Case reports (CARE)

Clinical practice guidelines (AGREE) and (RIGHT)

Qualitative research (SRQR) and (COREQ)

Animal pre-clinical studies (ARRIVE)

Quality improvement studies (SQUIRE)

Economic evaluations (CHEERS)

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Examples of statements to be used when ethics approval has been obtained:

• All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of A (No. ...).

• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

• Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

• Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

• This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

• This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

Examples of statements to be used when no ethical approval is required/exemption granted:

• This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

• The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed exempt by our XYZ Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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