

UNIVERSIDADE ESTADUAL DE CAMPINAS

UYRÁ DOS SANTOS ZAMA



"ESTUDO ESTRUTURAL E ULTRAESTRUTURAL DOS
ESPERMATOZÓIDES NAS TRIBOS APINI, BOMBINI, EUGLOSSINI E
MELIPONINI (HYMENOPTERA: APINAE), COM CONSIDERAÇÕES
FILOGENÉTICAS".

Este exemplar corresponde à redação final
da tese defendida pelo(a) candidato (a)
Uyrá dos Santos Zama
e aprovada pela Comissão Julgadora.

A handwritten signature in cursive script, likely belonging to the advisor, Mary Anne Heidi Dolder.

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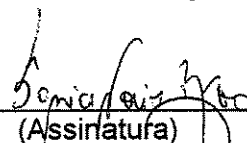
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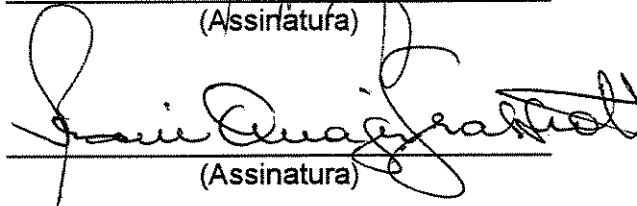
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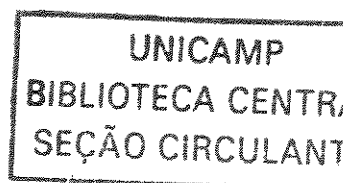
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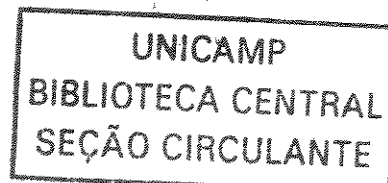
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Quando nasci um anjo esbelto,
desses que tocam trombeta, anunciou:
vai carregar bandeira !
Cargo muito pesado pra mulher,
esta espécie ainda envergonhada.
Aceito os subterfúgios que me cabem,
sem precisar mentir.
Não sou tão feia que não possa casar,
acho o Rio de Janeiro uma beleza e
ora sim, ora não, creio em parto sem dor.
Mas o que sinto escrevo. Cumpro a sina.
Inauguro linhagens, fundo reinos
_ dor não é amargura.
Minha tristeza não tem pedigree,
já a minha vontade de alegria,
sua raiz vai ao meu mil avô.
“Vai ser coxo na vida” é maldição pra homem.
Mulher é desdobrável.
Eu sou.

Com licença poética

Adélia Prado,
poetiza mineira



Dedico este trabalho àqueles que
me dedicam suas vidas,
meus pais, Acyr e Marly,
e meu grande amor, Gustavo.
Obrigada aos meus irmãos,
Pedro, Maíra e Mariana.

Agradeço ao Gustavo, meu amor,
pelo constante apoio,
compreensão e carinho nas minhas
mais diversas intempéries...
Muito obrigada pelo seu,
já imprescindível, amor.

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Cantiga triste, pode com ela
é quem não perdeu a alegria.

Toada,
Adélia Prado

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O termo “espermiocladística” defini a utilização de características estruturais e ultra-estruturais dos espermatozóides na composição de matrizes de caracteres para estudos filogenéticos. Estudos realizados, inclusive com insetos, têm fornecido dados consistentes e úteis para este fim, pelo menos para táxons superiores. Neste sentido, propusemos para este trabalho de tese, caracterizar detalhadamente a morfologia dos espermatozóides nas tribos Meliponini, Euglossini e Bombini, buscando identificar caracteres potenciais que pudessem contribuir para o entendimento das relações filogenéticas do grupo. Para tanto, utilizamos as metodologias usuais de (1) microscopia de luz, contraste de fase e fluorescência com DAPI e (2) Microscopia Eletrônica de Transmissão, convencional e citoquímica com ácido tânico e E-PTA. Ainda, comparamos nossos dados com o descrito na literatura para a tribo Apini, para que pudéssemos compor uma matriz consistente de caracteres. Nossas análises filogenéticas, embora incipientes, sugerem (Meliponini (Euglossini (Bombini + Apini))). Entretanto, acreditamos que nossos dados devam ser associados aos dados de morfologia somática, comportamento e biologia molecular, para que as análises filogenéticas futuras sejam mais conclusivas. Este trabalho originou 2 manuscritos em fase de submissão, um já aceito para publicação e um artigo já publicado: Zama, U., Lino-Neto, J. & Dolder, H. 2001 . Ultrastructure of spermatozoa in *Plebeia* (*Plebeia*) *droryana* Friese (Hymenoptera: Apidae: Meliponina). **Journal of Hymenoptera Research**, 10 (2): 261-270.

The term "spermiocladistics" express the use of structural and ultrastructural characteristics to compose character matrices for phylogenetic studies. Such studies, carried out for different animals, including insects, have been shown to be consistent and useful, at least for higher taxa. Therefore, we proposed, in this thesis, to carry out a detailed ultrastructural analysis of the spermatozoa of the tribes: Melliponini, Euglossini and Bombini, in order to identify characters that could potentially contribute to the understanding of phylogenetic relationships in this group. For this study, we employed the methods: (1) light microscopy using phase contrast, DAPI fluorescence and (2) transmission electron microscopy with conventional preparations and cytochemistry (E-PTA). Our data were compared with those described in the literature for the Apini tribe, so as to compose a consistent character matrix. Phylogenetic analysis of our data suggested (Meliponini (Euglossini (Bombini + Apini))). However, we believe that our data should be considered together with the data obtained with research on somatic morphology, behavior and molecular biology, in an effort to develop more consistent results. This thesis includes two manuscripts that are being submitted for publication, one was accepted and one article already published: : Zama, U., Lino-Neto, J. & Dolder, H. 2001 . Ultrastructure of spermatozoa in *Plebeia (Plebeia) droryana* Friese (Hymenoptera: Apidae: Meliponina). **Journal of Hymenoptera Research**, 10 (2): 261-270.

1. A ORDEM HYMENOPTERA – CLASSIFICAÇÃO

Os Hymenoptera constituem uma das quatro maiores ordens de insetos, com cerca de 15.000 espécies descritas. Estes são divididos em duas subordens, os Symphyta e os Apócrita (LaSalle & Gauld, 1992). Os Symphyta são considerados os Hymenoptera mais basais, caracterizados por uma ampla ligação do tórax ao abdome, ao contrário dos Apócrita que apresentam uma constrição característica separando o tórax fundido ao primeiro segmento do abdome, do restante deste (Borror, 1992).

Os Apócrita compreendem a grande maioria das espécies e são divididos em Aculeata e Parasítica (ou não-aculeata) (LaSalle & Gauld, 1992). Os Aculeata são o grupo mais conhecido e incluem as abelhas, formigas e vespas. Estes são considerados um grupo monofilético e o mais derivado dos Hymenoptera, sendo caracterizado fundamentalmente pela presença de ferrão nas fêmeas, desenvolvido a partir de modificações do ovipositor. O grupo Parasítica compreende as demais vespas Apócritas desprovidas de ferrão, com ovipositor funcional.

Os Apoidea são uma superfamília dos Apócrita, constituídos pelas famílias de abelhas, Stenotritidae, Colletidae, Andrenidae, Halictidae, Melittidae, Megachilidae e Apidae (Michener, 2000), além de quatro famílias de Sphecidae recém incluídas, sendo elas Heterogynaidae, Ampulicidae, Sphecidae *s.str.* e Crabronidae (Melo, 1999; Silveira et al., 2002). Adicionalmente, Alexandre e Michener (1995) reconhecem mais duas famílias de abelhas de língua curta, as Dasypodaidae e Meganomiidae.

A família Apidae é ainda dividida em três subfamílias: Xylocopinae, Nomadinae e Apinae. Finalmente, os Apinae compreendem 19 tribos (Michener, 2000), dentre as quais, quatro são ditas corbiculadas em referência ao aparato para o transporte de pólen, presente na superfície externa da tíbia posterior das operárias, chamado corbícula. Estas quatro tribos corbiculadas são os Apini, Bombini, Euglossini e Meliponini (Michener, 2000). É importante ressaltar que o reconhecimento dos corbiculados como quatro tribos distintas é uma indicação de Michener em seu trabalho com Roig-Alsina (Roig-Alsina & Michener, 1993) e Michener (2000). Entretanto, no mesmo trabalho, Roig-Alsina sugere apenas uma tribo Apini, constituída de quatro subtribos Apina, Bombina, Euglossina e Meliponina (Roig-Alsina & Michener, 1993). No presente trabalho, seguiremos portanto, a classificação proposta por Michener (2000):

Quadro de classificação das abelhas corbiculadas:

- Ordem: Hymenoptera
- Subordem: Apocrita (aculeata)
- Superfamília: Apoidea
- Família: Apidae
- Subfamília: Apinae
 - Tribo: Apini
 - Tribo: Bombini
 - Tribo: Euglossini
 - Tribo: Meliponini

2. ABELHAS CORBICULADAS - DIVERSIDADE E IMPORTÂNCIA ECONÔMICA E ECOLÓGICA

2.1. APINI

Esta tribo é representada por um único gênero, *Apis*, contendo 11 espécies (Michener, 2000). É um grupo originário das regiões tropicais da Ásia e África e inicialmente restrito ao Velho Mundo (Silveira et al., 2002). Entretanto, atualmente apresenta-se distribuído em todos os continentes pela introdução deliberada para produção de mel e polinização de culturas (Silveira et al., 2002). No Brasil, a introdução da espécie *Apis mellifera* Linnaeus, 1758, é bem documentada. Inicialmente, entre 1839 e 1874, colônias da raça européia foram introduzidas em diversos estados brasileiros, entretanto estas não teriam se adaptado bem às condições ambientais (relatos de Nogueira-Neto, 1972, *apud* Silveira et al., 2002). Porém, com a introdução da subespécie africana, *A. mellifera scutellata*, liberada acidentalmente em 1957 em Rio Claro, SP, é que se concretizou a disseminação e fixação de espécies híbridas em todo Brasil (Silveira et al., 2002).

Embora no Brasil os Apini sejam representados por apenas uma espécie, este grupo apresenta grande impacto econômico e ecológico. O grande interesse econômico refere-se principalmente à atividade de Apicultura, que constitui fundamentalmente o manejo de colmeias para extração de mel, própolis, geleia real, cera e outros produtos

das colmeias. Ainda, esta atividade é desenvolvida em associação ao plantio de diversas culturas, como por exemplo a citricultura, objetivando um aumento de produtividade pela polinização intensiva das flores, feita pelas abelhas.

O impacto ecológico da introdução da *A. mellifera* no ambiente das Américas foi largamente estudado tanto no que se refere à competição por alimento com as espécies nativas (Roubik, 1978, 1980, 1982), quanto ao efeito sobre a relação entre polinizadores e plantas nos ambientes naturais e sucesso reprodutivo das plantas nativas (Silveira et al., 2002).

2.2. BOMBINI (mamangabas sociais)

Esta tribo é constituída apenas pelo gênero *Bombus* Latreile, subdivididos em 37 subgêneros e cerca de 250 espécies (Michener, 2000). Este grupo ocorre primariamente nas áreas de baixas temperaturas da região Holártica, principalmente Eurásia (Hanson & Gauld, 1995; Silveira et al., 2002). No Brasil, ocorrem apenas seis espécies, todas do subgênero *Fervidobombus* Skorikov, sendo elas *atratus* Franklin, 1913; *bellicosus* Smith, 1879, *brasiliensis* Lepeletier, 1836; *brevivillus* Franklin, 1913; *morio* Swederus, 1787 e *transversalis* Olivier, 1789 (Silveira et al., 2002). Este grupo é geralmente reconhecido pela aparência robusta, com abelhas escuras e peludas, medindo cerca de 9 a 22 mm (Michener, 2000).

Os Bombini são polinizadores de grande importância econômica, tendo sido introduzidos em diversas culturas, tanto no campo quanto em casas de vegetação, com objetivo de aumentar a produtividade. Inclusive, Silveira et al. (2002) sugere a provável entrada de *Bombus ruderatus* Fabricius em território brasileiro, em função de sua introdução no Chile na década de 80 para a polinização de trevo branco (*Trifolium repens*). Esta espécie se estabeleceu e ampliou sua distribuição no Chile, Argentina e região oeste da Patagônia (Roig-Alsina & Aizen, 1993) e, ainda segundo Silveira et al. (2002), só não penetrará no Brasil, se esta se mostrar intolerante ao clima quente.

2.3. EUGLOSSINI

Esta tribo é constituída por cinco gêneros, sendo eles *Euglossa*, *Eulaema*, *Eufriesea*, *Exaerete* e *Aglae*, compreendendo cerca de 175 espécies (Michener, 2000). Os Euglossini são amplamente distribuídos na região tropical, sendo mais diversificados nas florestas úmidas (Silveira et al., 2002).

Os Euglossini variam de médio a grande porte, com cerca de 8,5 a 29 mm. Algumas destas abelhas são reconhecidas por apresentarem uma coloração marcante em tons de azul, verde, violeta e cobre, com nuances metálicas, enquanto outras são robustas, escuras e bastante peludas (Kimsey, 1987; Hanson & Gauld, 1995; Silveira et al., 2002). Outra importante característica é a presença de uma língua longa (proboscide), que em repouso, alcança pelo menos a base do metassoma (Hanson & Gauld, 1995; Silveira et al., 2002).

Um comportamento bastante interessante dos Euglossini, é o fato dos machos coletarem substâncias aromáticas em flores principalmente de Orchidaceae, Araceae, Gesneriaceae e Solanaceae, além de também aproveitarem substâncias produzidas por fungos e outras fontes (ver Rebêlo, 2001; Silveira et al., 2002). A coleta destas substâncias possivelmente está relacionada a aspectos de biologia reprodutiva, como por exemplo, para serem aproveitadas após transformações metabólicas, como atrativos para as fêmeas (Hanson & Gauld, 1995). Em função da grande associação destas abelhas com as orquídeas, o grupo recebeu a denominação genérica de abelhas das orquídeas (orchid bees) (Hanson & Gauld, 1995; Michener, 2000), embora polinizem também os demais grupos de plantas supracitados.

2.4. MELIPONINI

Os Meliponini constituem o grupo mais diverso dentre os corbiculados, onde Silveira et al. (2002) reconhecem 27 gêneros, com várias centenas de espécies, sendo que o número total dificilmente será encontrado, em função da grande quantidade de espécies crípticas (Michener, 2000).

Esta tribo está distribuída em todas as regiões tropicais do mundo, bem como nas regiões subtropicais do hemisfério sul (Michener, 2000; Silveira et al., 2002).

As abelhas desta tribo são de pequeno a médio porte, medindo cerca de 1,8 a 13,5 mm, sendo fundamentalmente caracterizadas pela atrofia do ferrão e estruturas associadas nas operárias (Stingless bees) (Michener, 1990, 2000).

Este grupo tem um grande impacto econômico e ecológico nos trópicos. Semelhante ao descrito para Apini, as colmeias de diversas espécies de Meliponini também são utilizadas na Meliponicultura, para extração de derivados. Entretanto, o maior interesse advém do fato de serem o mais importante grupo de polinizadores, muitas vezes restritos, das fanerógamas tropicais (Bawa et al., 1985), sendo estas tanto espécies vegetais nativas ou cultivadas (Hanson & Gauld, 1995). Neste sentido, diversos estudos já

foram realizados buscando estabelecer as relações entre espécies de abelhas e espécies cultivadas (Michelbacher et al., 1971; Wille, 1976; Wille et al., 1983; O'Toole, 1993), bem como buscando inferir a importância do grupo para a manutenção da diversidade de espécies vegetais nativas (Neff & Simpson, 1993). Embora na maioria das vezes as abelhas nativas “sem-ferrão” sejam benéficas às atividades agrícolas, em determinadas condições algumas espécies podem se tornar pragas, como por exemplo, *Trigona corvina*, *T. fuscipennis*, *T. silvestriana* e *T. nigerrima* que cortam as flores em plantações de citrus, macadamia, manga, entre outras (Wille, 1965; Camacho, 1966; Haueisen Freire & Gara, 1970). E, finalmente, foi relatado que algumas abelhas podem transmitir patógenos enquanto visitam as flores (Buddenhagen & Elsasser, 1962).

3. COMPORTAMENTO SOCIAL

Os Hymenoptera são um grupo muito diverso em termos de comportamento social. A grande maioria de seus membros são solitários. Entretanto, diferentes níveis de organização social são encontrados, em níveis que variam desde gregários, subsociais, parassociais (comunais, quasissociais, semissociais) até culminarem nos eussociais e eussociais avançados.

Resumidamente, a definição dos diferentes níveis de organização social são baseados nos seguintes parâmetros (Michener, 1974):

	Castas e divisão de trabalho	Colônias com adultos de 2 gerações	Cooperação no trabalho nas células	Fêmeas morfologicamente similares	Alimentação progressiva
Subsocial	-	-	-	+	+
Parassocial:					
• Communal	-	-	-	+	-
• Quasissocial	-	+/-	+	+	+/-
• Semissocial	+	-	+	+	+/-
Eussocial:					
• primitivo	+	+	+	+	+/-
• avançado	+	+	+	-	+/-

(+ presença; - ausência; +/- variável)

Considerando a sociabilidade, as quatro tribos corbiculadas são o grupo mais diverso dentre os Hymenoptera, sendo que seus membros apresentam todas as variedades de comportamento social.

Em Euglossini, a maioria das espécies são solitárias, representando os gêneros *Euglossa* e *Eufriesea*, entretanto algumas espécies de *Eufriesea* produzem agregados de fêmeas em locais protegidos (Michener, 2000). Ainda, pelo menos alguns *Euglossa* e provavelmente todos *Eulaema*, constituem colônias parassociais com várias fêmeas, enquanto que os gêneros *Aglae* e *Exaerete* são cleptoparasitas de ninhos de *Eulaema* e *Eufriesea* (Michener, 2000; Silveira et al., 2002).

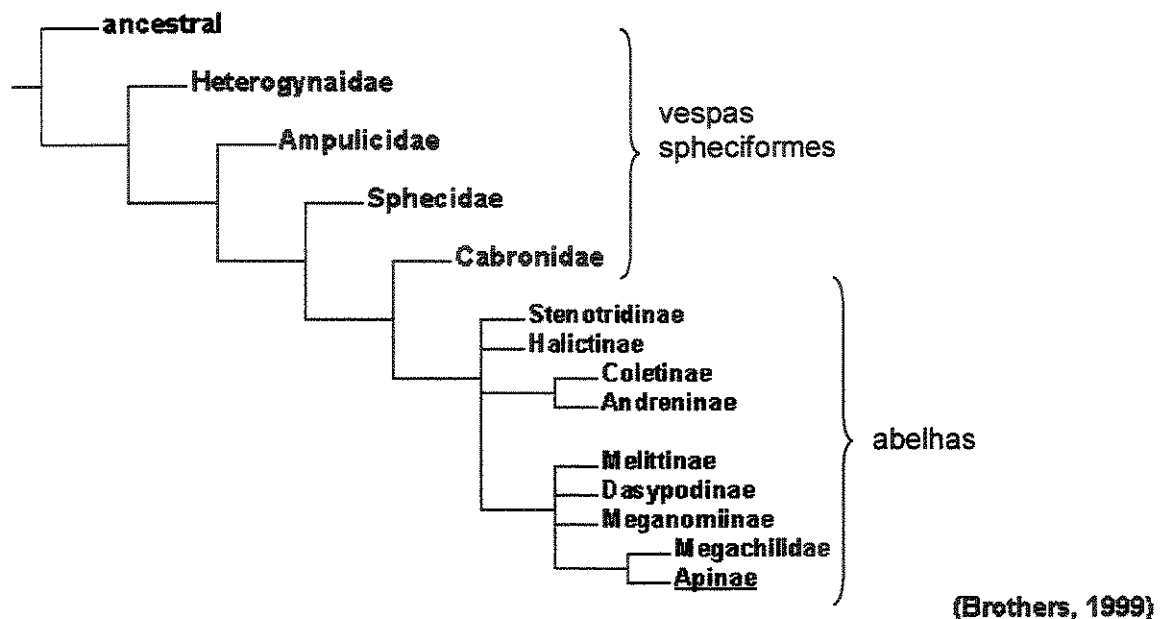
Em Bombini, a grande maioria das espécies formam colônias eussociais primitivas, exceto em ninhos observados no começo da primavera onde a rainha (fundadora) é quem cuida dos imaturos (subsocial) (Michener, 2000). Michener (2000), inclui ainda no gênero *Bombus*, a única espécie parasita da tribo, classificada até então como um gênero separado *Psithyrus* Lepeletier (Silveira et al., 2002).

Nas tribos Apini e Meliponini, todas as espécies formam colônias perenes eussociais avançadas, embora alguns meliponíneos vivam com alimentos roubados de outras espécies (Silveira et al., 2002). Além das abelhas, o comportamento eussocial avançado só foi observado em formigas (Hymenoptera: Vespoidea: Formicidae) e em cupins (Isoptera) (Borror, 1992).

4. FILOGENIA DOS CORBICULADOS

A Filogenia nos Hymenoptera ainda é bastante controversa, embora diversos estudos tenham sido produzidos enfocando independentemente tanto características morfológicas e comportamentais, quanto moleculares (ex. Alexander, 1991, 1992; Whitfield, 1992; Brothers, 1999; Carpenter & Wheeler, 1999; Dowton & Austin, 1994, 1999; Melo, 1999; Ronquist, 1999; Noll, 2002). Ainda, a carência de consenso é observada tanto nos táxons superiores da ordem, quanto nos grupos menores. Controvérsias podem ser observadas até entre os grupos mais bem estudados como os Aculeata.

Atualmente, a subfamília Apinae é posicionada como um grupo bastante derivado dos Apoidea, sendo as quatro subfamílias de vespas spheciformes, considerados os grupos mais basais (Brothers, 1999; Melo, 1999):



Dentro dos Apinae, parece consensual o posicionamento dos corbiculados como os mais derivados da subfamília. Entretanto, as relações entre as quatro tribos constituintes, ainda permanecem controversas (Michener, 1944, 1990; Winston & Michener, 1977; Kimsey, 1984; Plant & Paulus, 1987; Sheppard & McPheron, 1991; Cameron, 1991, 1993; Prentice, 1991; Roig-Alsina & Michener, 1993; Chavarria & Carpenter, 1994; Mardulyn & Cameron, 1999; Koulianos et al., 1999; Cameron & Mardulyn, 2001; Silveira et al., 2002; Noll, 2002). Estes estudos foram fundamentados em caracteres morfológicos, moleculares e comportamentais, no entanto, as hipóteses filogenéticas para os corbiculados são bastante diversas:

1. **(EUGLOSSINI (BOMBINI (MELIPONINI + APINI))**: sustentada por caracteres morfológicos (Michener, 1944; Prentice, 1991; Roig-Alsina & Michener, 1993), comportamentais (Noll, 2002) e pela análise de "Evidência Total" que associou dados morfológicos aos moleculares (Chavarria & Carpenter, 1994);
2. **(APINI (EUGLOSSINI (MELIPONINI + BOMBINI))**: sustentada por caracteres moleculares (Cameron, 1993; Cameron & Mardulyn, 2001; Koulianos et al., 1999; Mardulyn & Cameron, 1999; Sheppard & McPheron, 1991);

3. **(MELIPONINI (APINI (BOMBINI + EUGLOSSINI):** sustentada por caracteres morfológicos (Winston & Michener, 1977; Kimsey, 1984);
4. **(EUGLOSSINI + APINI) (MELIPONINI + BOMBINI):** sustentada por caracteres moleculares (Cameron, 1991);
5. **(EUGLOSSINI + BOMBINI) (MELIPONINI + APINI):** sustentada por caracteres morfológicos (Michener, 1990);
6. **(EUGLOSSINI (MELIPONINI (BOMBINI + APINI):** sustentada por caracteres morfológicos (Plant & Paulus, 1987).

Provavelmente a maioria das controvérsias ou dúvidas a respeito da filogenia dos Hymenoptera como um todo, ou de grupos específicos, somente serão resolvidas com análises conjuntas incluindo novos sistemas de caracteres, especialmente aqueles obtidos a partir de estruturas menos susceptíveis às condições ambientais.

Nesse sentido, a análise filogenética usando características morfológicas de estruturas internas, como por exemplo os espermatozoides, pode fornecer uma fonte de dados não tradicionais, tendo seus caracteres uma natureza mais conservativa que os caracteres morfológicos usados tradicionalmente, razão pela qual a morfologia do espermatozoide tem sido empregada para resolver muitos problemas filogenéticos (espermiocladística) e taxonômicos em vários grupos animais, incluindo os insetos (Dallai, 1979; Baccetti, 1987; Jamieson, 1987; Carcupino et al., 1995; Dallai & Afzelius, 1995; Jamieson et al., 1999).

5. ULTRAESTRUTURA DOS ESPERMATOZÓIDES EM HYMENOPTERA

Atualmente os espermatozoides em Hymenoptera só foram descritos em detalhes nos Symphyta (Quicke et al., 1992; Newman & Quicke, 1999a), nas abelhas *Apis mellifera* (Rothschild, 1955; Hoage & Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Woyke, 1984; Peng et al., 1992, 1993; Lino-Neto et al., 2000b), nos Meliponini (Zama et al., 2001, 2003), em alguns Formicidae (Thompson & Blum, 1967; Caetano, 1980; Wheeler et al., 1990; Lino-Neto & Dolder, 2002), em alguns Chalcidoidea (Lee & Wilkes, 1965; Wilkes & Lee, 1965; Hogge & King, 1975; Lingmei & Dunsu, 1987; Lino-Neto et al.,

1999, 2000a; Lino-Neto & Dolder, 2001b), em algumas espécies de Ichneumonoidea (Chauvin et al., 1988; Quicke et al., 1992), em *Leptopilina heterotoma*, Cynipoidea (Quicke et al., 1992; Newman & Quicke, 1999b), em *Megalyra fasciipennis*, Megalyroidea (Newman & Quicke, 2000) e em *Trissolcus basalís* e *Telenomus podisi*, Scelionidae (Lino-Neto & Dolder, 2001a). Apesar das publicações serem restritas a algumas espécies e portanto, serem pouco representativas se considerarmos a grande diversidade dos Hymenoptera, algumas características ultraestruturais parecem comuns para a ordem.

Os espermatozóides nos Hymenoptera são células finas e alongadas, constituídas por uma região de cabeça e outra de flagelo. A região de cabeça compreende geralmente o acrosso e o núcleo, enquanto que a região de flagelo é constituída pelo axonema com padrão 9+9+2 microtúbulos; geralmente, dois derivados mitocondriais; um longo adjunto do centríolo e dois corpos acessórios.

Esta caracterização geral apresenta uma série de modificações nos diferentes grupos de Hymenoptera, fornecendo diversidade suficiente para justificar estudos descritivos, que possam dar suporte a futuros estudos de taxonomia, sistemática, biologia reprodutiva e filogenia.

Este trabalho teve como objetivos específicos:

- (1) descrever a estrutura e ultraestrutura dos espermatozóides em representantes das tribos Meliponini, Euglossini e Bombini (Hymenoptera: Apinae);
- (2) comparar a morfologia dos espermatozóides de Meliponini, Euglossini e Bombini, com o descrito para Apini;
- (3) avaliar a utilização dos espermatozóides nestas tribos para fornecimento de caracteres consistentes para estudos filogenéticos;
- (4) propor uma hipótese filogenética para as tribos corbiculadas dos Apinae, segundo nossos dados.

Como objetivo geral, este trabalho foi idealizado no sentido de contribuir para o conhecimento da biologia e taxonomia das espécies de Apinae, bem como dar suporte para futuros estudos filogenéticos nessa subfamília.

TRIBO APINI

A morfologia dos espermatozóides de *Apis mellifera*, o único Apini de ocorrência no Brasil, já é bastante conhecida. Nossas análises comparativas são baseadas em oito trabalhos publicados entre 1955 e 2000, sendo eles:

Rothschild, L. 1955. The spermatozoa of the honey bee. **Trans. R. Ent. Soc. Lond.**, **107**: 289-294.

Hoage, T. R. & Kessel, R. G. 1968. An electron microscope study of the process of differentiation during spermatogenesis in the drone honey bee (*Apis mellifera* L.) with special reference to centriole replication and elimination. **J. Ultrastruc. Res.**, **24**: 6-32.

Cruz-Höfling, M. A., Cruz-Landim, C. & Kitajima, E. W. 1970. The fine structure of spermatozoa from the honey bee. **An. Acad. Brás. Ciênc.**, **42**: 69-78.

Lensky, Y., Ben-David, E. & Schindler, H. 1979. Ultrastructure of the spermatozoan of the mature drone honey bee. **J. Apic. Res.**, **18**: 264-271.

Woyke, J. 1984. Ultrastructure of single and multiple diploid honey bee spermatozoa. **J. Hym. Res.**, **23**: 123-135.

Peng, C. Y. S., Yin, C. M. & Yin, L. R. S. 1992. Effect of rapid freezing and thawing on cellular integrity of honey bee sperm. **Physiological Entomology**, **17**: 269-276.

Peng, C. Y. S., Yin, C. M. & Yin, L. R. S. 1993. Ultrastructure of honey bee, *Apis mellifera* sperm with special emphasis on the acrosomal complex following high-pressure freezing fixation. **Physiological Entomology**, **18**: 93-101.

Lino-Neto, J., Bão, S. N. & Dolder, H. 2000. Sperm ultrastructure of the honey bee (*Apis mellifera*) (L) (Hymenoptera, Apidae) with emphasis on the nucleus-flagellum transition region. **Tissue & Cell**, **32**: 322-327.

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Ultrastructure of Spermatozoa in *Plebeia (Plebeia) droryana* Friese (Hymenoptera: Apidae: Meliponina)

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Abstract.—In general, the spermatozoa of *Plebeia (Plebeia) droryana* Friese are very similar to those described for other Hymenoptera. However, their arrangement in spermatodesmata bundles in the seminal vesicle has not yet been found in Apidae, this being a characteristic observed, to date, only in Symphyta, the Hymenoptera considered most primitive. The spermatozoa are long and thin, made up of a head connected to the tail at the position of the centriolar adjunct. The head includes an acrosomal vesicle, a perforatorium and a electron dense nucleus. The flagellum consists in a typical axoneme, two mitochondrial derivatives and two accessory bodies. Unlike most other Hymenoptera, the centriolar adjunct is very long and located between the nuclear base and the anterior extremity of the smaller mitochondrial derivative. It has recently been demonstrated that the structure and ultrastructure of hymenopteran spermatozoa are sufficiently varied so as to furnish consistent character matrices that can contribute to phylogenetic studies ("Spermiocladistics"). Since no consensual phylogenetic hypothesis has yet been proposed for Apidae, the data presented here may be a contribution in this direction.

The Apidae have been extensively studied due to their economic and ecological importance, since they are pollinators, often exclusively, of the majority of flowering plants, including species cultivated by man. The relation between these pollinating agents and the plants they pollinate is so intimate that changes in the biodiversity of either group is certain to affect the other. The Apidae are also recognized as a diverse group with complex social behaviour, which culminate in advanced eusocial societies, a level observed only among Hymenoptera (a few bees and wasps) and in the Isoptera.

Within the Apidae, the tribe Apini, consisting in the subtribes Apina, Bombina, Euglossina and Meliponina (*sensu* Roig-Alsina and Michener 1993), is particularly interesting because its members collectively display all levels of social behaviour. Ranging from solitary bees, as in some Euglossina, to advanced eusocial groups,

such as the Apina and the Meliponina, passing through intermediate social behavior groupings as found in the Bombina and Euglossina.

In spite of the unquestioned importance of the Apidae, so far neither morphological nor molecular studies have been able to establish an uncontested phylogeny for this group (Camargo and Pedro 1992b; Cameron 1991, 1993; Cameron et al. 1992). The establishment of the phylogeny of this group would undoubtedly be important for studies of the evolutionary mechanism, or mechanisms, leading to eusocial behaviour (Crozier and Pamilo 1996).

Structural and ultrastructural characteristics of the spermatozoa, besides their own biological and taxonomic aspects, may be very interesting if this information can be used to form a character matrix for phylogenetic analysis. This information, associated with other character systems, could lead to a better understanding of the

evolutionary relationships within the group ("spermiocladistics", Jamieson 1987) as is being carried out for other animals, including insects (Baccetti 1972; Dallai 1979; Dallai and Afzelius 1990, 1995; Carcupino et al. 1995; Jamieson et al. 1999; Lino-Neto et al. 1999, 2000a, 2000b).

The spermatozoal ultrastructure of only one apid species, *Apis mellifera* Linnaeus, representing the Apini, has so far been studied in detail (Rothschild 1955; Hoage and Kessel 1968; Cruz-Höfing et al. 1970; Lensky et al. 1979; Woyke 1970; Lino-Neto et al. 2000b). Besides this species, in Meliponina only some aspects of spermiogenesis were investigated, including that of *Scaptotrigona postica* Latreille (Cruz-Landim and Beig 1980; Cruz-Landim et al. 1980), *Melipona quadrifasciata anthidioides* Lepeletier (Cruz-Landim et al. 1980; Cruz-Landim and Moraes 1980), *Plebeia (Plebeia) droryana* Friese, *Frieseomelitta (Frieseomelitta) varia* Lepeletier, *Leurotrigona muelleri* Friese (Cruz-Landim et al. 1980). However these publications contain almost no information on the mature sperm cell. Therefore, in this study, we characterize the structure and ultrastructure of *Plebeia (Plebeia) droryana* sperm so as to furnish data that could be used for future phylogenetic research.

MATERIAL AND METHODS

Adult males of *Plebeia (Plebeia) droryana* were obtained from colonies maintained in the Central Apiary of the Federal University of Viçosa, MG, Brazil.

Light Microscopy.—Seminal vesicles were dissected and broken open on clean glass microscope slides, where the sperm were spread and fixed in a solution of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60), equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min. with 0.2 µg/ml 4,6-diamino-2-phenylindole

(DAPI) in phosphate buffered saline, washed, and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360–370 nm excitation filter.

Transmission Electron Microscopy.—Seminal vesicles were dissected and fixed for 3 hours in a solution containing 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.2. The materials were post fixed in 1% osmium tetroxide, in the same buffer, for 1–2 hours. Dehydration was carried out in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with the Zeiss LEO 906 transmission electron microscope.

RESULTS

In the seminal vesicle, the spermatozoa of *Plebeia (Plebeia) droryana* are organized in spermatodesmata bundles, where the anterior region of the heads are embedded in a substance of medium electron density (Figs. 1, 2). The more central spermatozoa are situated slightly ahead of the lateral ones, so that a transverse section of this region shows acrosomes sectioned at different levels (Fig. 2). However, some isolated spermatozoa also appear chaotically dispersed in the seminal vesicles (Figs. 5–7).

The spermatozoan of *P. droryana* is long and thin, measuring approximately 135 µm in length (Fig. 3). The acrosome measuring about 1.2 µm and is made up of the acrosomal vesicle and the perforatorium (Figs. 1, 5–6). The acrosomal vesicle is cone-shaped and covers the perforatorium along its entire length (Fig. 6). In transverse section, the acrosome is circular at the tip but becomes triangular, particularly the perforatorium towards the nucleus (Figs. 2, 8–9). Along the circular portion, an electron transparent layer covers the perforatorium, separating it completely from the acrosomal vesicle. However,

when they are triangular this clear layer is reduced to patches at the vertices (Figs. 8–9). The perforatorium base penetrates about 70 nm into a small asymmetric cavity in the nuclear tip (Fig. 7).

The nucleus measures approximately 7.5 μm in length and is filled homogeneously with dense chromatin. In transverse section, it is slightly oval, measuring approximately 0.18 μm in diameter at the anterior extremity and 0.45 μm at the posterior (Figs. 2–7, 10–13). At the anterior tip there is a cavity in which the perforatorium fits (Fig. 7), while posteriorly the nucleus tapers conically and is covered by thin electron transparent and electron dense material (Figs. 12–13, 15).

The axoneme, measuring 126 μm of length, presents the 9+9+2 pattern of microtubules, with 9 single, external, accessory microtubules, nine doublets and a pair of single ones in the center of the arrangement (Figs. 18–21). In the first 0.28 μm , corresponding to the centriole, the axoneme consists only of the accessory microtubules, the doublets and a dense amorphous substance (Fig. 16). The central microtubules begin posterior to the centriolar portion (Fig. 17). In the final portion, the axoneme is gradually disorganized, with the central microtubules and the nine doublets terminating first, simultaneously, followed by the accessory microtubules (Figs. 21–24).

The centriolar adjunct is very long, about 4.6 μm in length, compact and electron dense. It begins at the nuclear base and extends parallel to the axoneme until it fits onto the smaller mitochondrial derivative (Figs. 11–14). In longitudinal section, it has a rod-like shape while in transverse section it is approximately circular, with a diameter of about 0.2 μm (Figs. 1, 11–12, 14, 16–18).

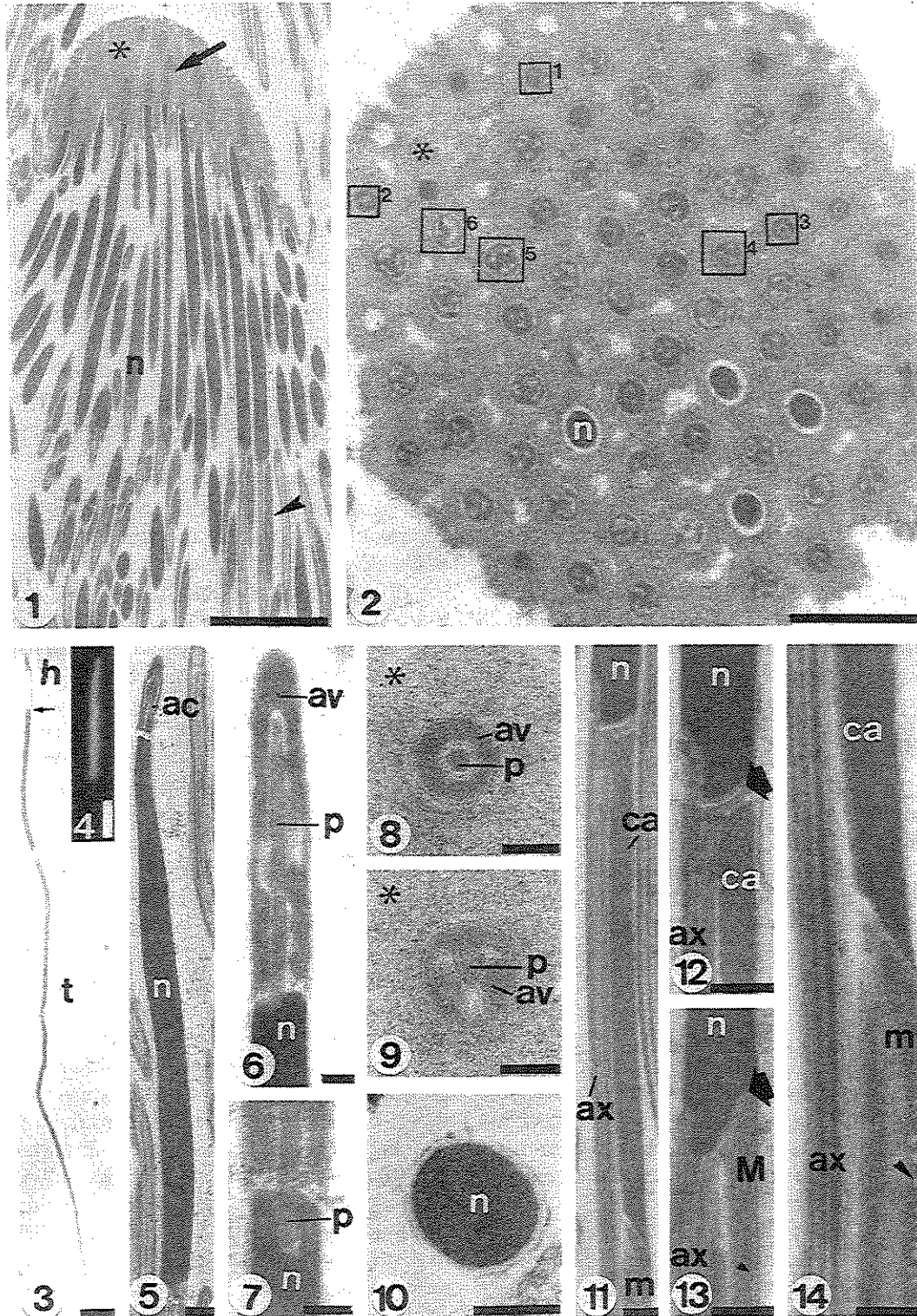
The mitochondrial derivatives are asymmetric in both length and diameter (Figs. 11, 13–14, 19). Anteriorly, the larger mitochondrial derivative begins next to the tapering nucleus (Fig. 13) and the

smaller in contact with the posterior end of the centriolar adjunct. In transverse section, the derivatives are elipsoidal, with the larger one curving slightly over the smaller one (Fig. 19). Both have at least three regions: a dense material that fills in most of the mitochondrial derivatives; a clear approximately central area and the region of the cristae, limited to that part of the periphery opposite the axoneme (a, b and c in Figs. 16–19). The large mitochondrial derivative also has a region of regularly arranged paracrystalline material in the third that is most distal to the axoneme (p in Figs. 16–19). Anteriorly, the derivative extremities do not show any cristae (Figs. 13–14).

The accessory bodies are located laterally, between the axoneme and the mitochondrial derivatives. In transverse sections, they have a triangular shape (Figs. 18–20). In the centriolar adjunct region, there is only one accessory body present between the larger mitochondrial derivative and the axoneme (Fig. 18).

DISCUSSION

The arrangement of spermatozoa in spermatodesmata observed in *Plebeia droryana*, has not been described for Apocrita. According to Quicke et al. (1992), this spermatozoa arrangement in bundles is characteristic of Symphyta, considered primitive Hymenoptera, in spite of some sheath fragments encountered by these authors in some Aculeata. The central spermatozoa of the sheaths are somewhat ahead of the others, as observed in *P. droryana*, as also occurs in Xyeloidea and Phamphiloidea, which are considered the most basal Symphyta (Newman and Quicke 1999a). However, in the Siricidae, considered the family most closest to Aculeata studied so far, the central spermatozoa are inserted well ahead of the peripheral ones, so that in transverse sections, they are observed in very different levels (Newman and Quicke 1999a). Although most of the spermatozoa are organized in



spermatodesmata in *P. droryana*, as in the symphytans, *Tremex* sp. (Newman and Quicke 1999a) and *Calameuta* sp. (Quicke et al. 1992), some spermatozoa are free. Newman and Quicke (1999a) suggested that the observation of free spermatozoa in the seminal vesicle could be due to fixation or if they indicate a pre-transfer phenomenon. We believe that it is also possible that these spermatozoa have either not yet been grouped into spermatodesmata, or even that not all spermatozoa are destined to become included in bundles.

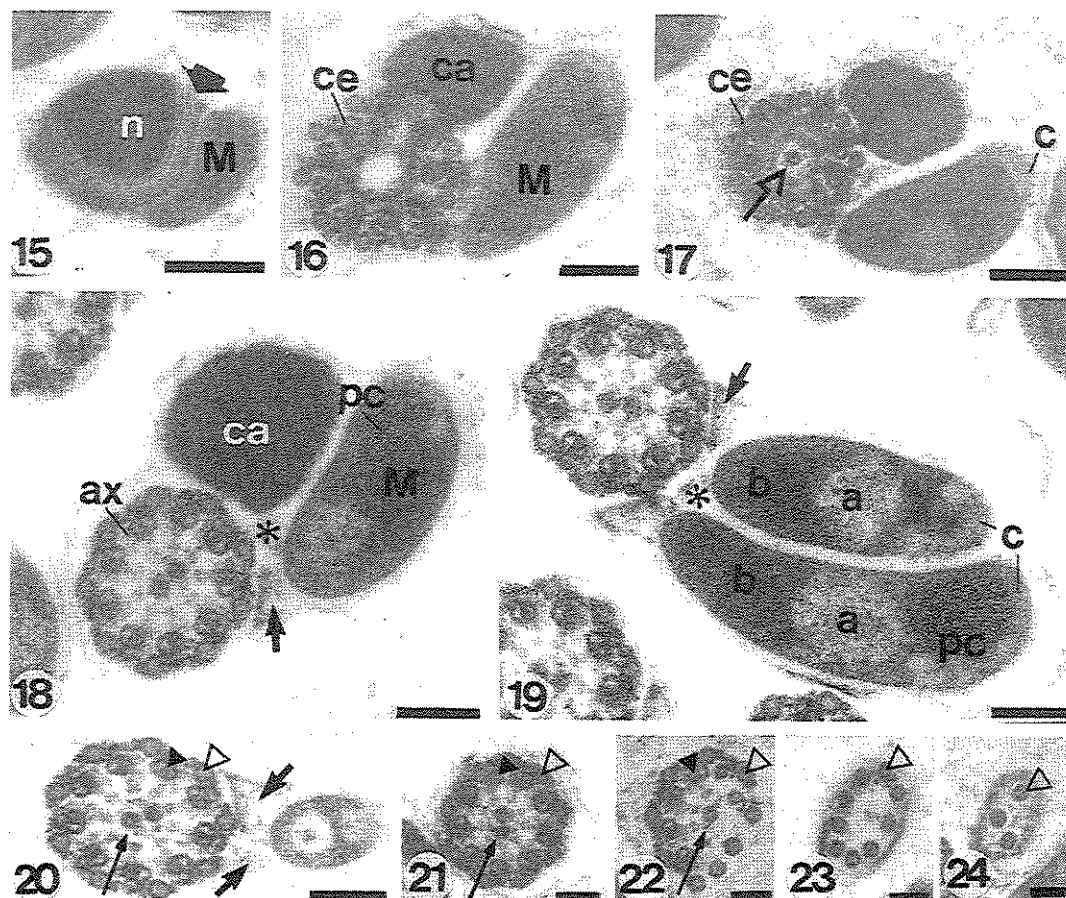
In all the apocritan non-Aculeata (parasitic wasps) considered to date, the spermatozoa appear isolated in the seminal vesicle, and no spermatodesmata fragments have been observed (ex. Quicke et al. 1992; Newman and Quicke 1998, 1999b; Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001a, b). The fact that spermatozoa organized in spermatodesmata occur in Symphyta and in at least one apocritan Aculeata, which are considered, respectively, the most basal and the most derived hymenopteran groups, while not occurring in the apocritan non-Aculeata, is very intriguing. This suggests either that it could be a reversed character state in Aculeata or that this group derived directly from the Symphyta, as is believed to have occurred with parasitic wasps. This latter hypothesis seems less likely

since morphological and molecular analyses suggest that Aculeata are the sister group of the Ichneumonoidea (Whitfield and Cameron 1998; Ronquist et al. 1999).

The basic structure of the spermatozoa in *P. droryana* is quite similar to that described for other Hymenoptera, as well as for insects in general (Phillips 1970; Baccetti 1972). The acrosome of *P. droryana*, made up of an acrosomal vesicle and the perforatorium appears to be typical for Hymenoptera (Jamieson 1999), having been found in Symphyta (Quicke et al. 1992; Newman and Quicke 1999a), in the Scelionidae, *Trissolcus basalis* (Lino-Neto and Dolder 2000a), in Formicidae (Wheeler et al. 1990) and in *Apis mellifera* (Cruz-Höfling et al. 1970; Hoage and Kessel 1968; Lensky et al. 1979; Peng et al. 1992, 1993). In this last species, unlike the other Hymenoptera studied, the acrosome is almost as long as the nucleus, measuring about 5.6 μm . The fact that the acrosome of *P. droryana* shows a circular cross section at the tip gradually being modified into a triangular form as it reaches towards the nucleus differs from other Hymenoptera where this acrosome are always circular (ex. Symphyta, Newman and Quicke 1999a; Cynipoidea, Newman and Quicke 1999b; Chalcidoidea, Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b; Formicidae, Wheeler 1990),

←

Figs. 1–14. Ultramicrographs of *Plebeia* spermatozoa in seminal vesicle. 1–2, Longitudinal and transverse sections, respectively, of anterior region of a spermatodesm. 1, Acrosomal region (arrow) and portion of nucleus (n) embedded in less electron dense extracellular material (*). The arrowhead indicates the centriolar adjunct. 2, Numbers 1–6 indicate acrosomes sectioned in anterior-posterior levels from tip to just above nucleus. 3–4, Phase contrast micrograph of a spermatozoa (3) and head region, DAPI-stained fluorescence of nucleus. The arrow indicates the head (h) and tail (t) limit. 5, Longitudinal section showing acrosome (ac) and nucleus. 6, Longitudinal section of acrosomal vesicle (av) and perforatorium (p). 7, Transition region of acrosome-nucleus showing perforatorium base fitting into cavity of nuclear tip. 8–10, Transverse section of acrosome tip (8), base of acrosome (9) and nucleus free of extra cellular material (10). 11–13, Longitudinal sections of nucleus-flagellum transition region. Arrows indicate connective material at nuclear base (12, 13); 14, Longitudinal section at junction of centriolar adjunct and smaller mitochondrial derivative. Arrowhead indicates mitochondrial cristae. Abbreviations: n = nucleus; ac = acrosome; av = acrosomal vesicle; p = perforatorium; ca = centriolar adjunct; ax = axoneme; M = larger mitochondrial derivative; m = smaller mitochondrial derivative. Scale bar: 1, 4, 8–9 = 3 μm ; 2 = 2 μm ; 3 = 8 μm ; 6 = 0.1 μm ; 7 = 0.2 μm ; 10–11, 14 = 0.3 μm and 5, 12–13 = 0.5 μm .



Figs. 15–24. Sequential transverse sections of flagella. 15, Nucleus-flagellum transition region. Arrow indicates material connecting nucleus to larger mitochondrial derivative. 16–17, Centriolar region of axoneme. Open arrow indicates first of central microtubules. 18–19, Sections of flagellum, at centriolar adjunct region and at both mitochondrial derivatives, respectively. The arrows indicate accessory bodies and (*) indicates central material between flagellar structures. 20–24, Final flagellar region. The nine doublets (arrowheads) and two central microtubules (small arrow) terminate first, followed by accessory ones (white arrowheads). Large arrows indicate accessory bodies. Abbreviations: a = less electron dense amorphous region; b = more electron dense amorphous region; c = cristae region; pc = paracrystalline region in the larger mitochondrial derivative; ca = centriolar adjunct; ce = centriole; n = nucleus; ax = axoneme. Scale bar: 15–20 = 0,1 μ m; 21–22 = 0,06 μ m and 23–24 = 0,05 μ m.

or maintains an oval cross section as in *Apis mellifera* (q.v.) and Vespidae (personal observation). The acrosome of *A. mellifera* also differs from that of *P. droryana* due to the presence of a long anterior projection of the acrosomal vesicle (Cruz-Höfling et al. 1970; Hoage and Kessel 1968). The penetration of the perforatorium in the nuclear tip as occurs in *P. droryana* has been described for the majority of the hymenopterans (ex. Quicke et al. 1992; Newman

and Quicke 1999a; Wheeler et al. 1990). However, in Eurytomidae, *Bephratelloides pomorum* Fabricius (Lino-Neto et al. 1999), and in the Pteromalidae, *Nasonia vitripennis* Walker (Hogge and King 1975), the perforatorium base is concave and has the same diameter as the nucleus in this region, fitting directly onto the anterior nuclear surface. In the majority of parasitic wasps, there is a third extracellular layer (the extracellular sheath), covering all of

the acrosome and extending along a variable length of the nucleus (Quicke et al. 1992; Newman and Quicke 1999b; Quicke et al. 1992; Newman and Quicke 1998; Quicke et al. 1992; Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b). Also, in some of these, the extra-cellular sheath gives rise to innumerable filaments, probably representing a well developed glycocalyx (Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b).

In *P. droryana*, the nucleus is long, dense and usually appears homogeneously compacted. These characteristics are highly conserved in Hymenoptera, and the variations observed have been in length and in the fact that this structure may be linear (ex. Quicke et al. 1992; Jamieson et al. 1999; Wheeler et al. 1990; Lino-Neto et al. 2000b), or twisted in a spiral, as in Chalcidoidea (Lee and Wilkes 1965; Hogge and King 1975; Quicke et al. 1992; Lino-Neto et al. 1999, 2000a, 2000b; Lino-Neto and Dolder 2001b), Scelionidae (Lino-Neto and Dolder 2001a) and Diapriidae (Quicke, personal communication). The nucleus of *P. droryana* ends in a short cone, next to the anterior tip of the large mitochondrial derivative, and terminating in contact with the centriolar adjunct and axoneme. In *Apis mellifera*, the final nuclear projection is considerably longer and inserted in the axoneme, so that in cross section the nucleus is found surrounding the tips of the centriolar microtubules (Peng et al. 1993; Lino-Neto et al. 2000b). In the majority of the Hymenoptera, the nucleus is not tapered posteriorly but instead is abruptly truncated (Quicke et al. 1992; Newman and Quicke 1999a; Newman and Quicke 1999b; Quicke et al. 1992; Wheeler et al. 1990; Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b).

The centriolar adjunct of *P. droryana* is a well developed structure located parallel to the axoneme and between the nucleus and the smaller mitochondrial derivative. This arrangement has also been found in some Symphyta (Newman and

Quicke 1999a), Cynipoidea (Newman and Quicke 1999b), Ichneumonoidea (Quicke et al. 1998) and in *A. mellifera* (Lino-Neto et al. 2000b). However, in the Ichneumonoidea this structure is comparatively short (Quicke et al. 1998) while in *A. mellifera*, it is extremely long, tapered anteriorly, widening into a thick rod posteriorly (Lino-Neto et al. 2000b). In the symphytan *Tremex* sp. (Newman and Quicke 1999a) and in the Formicidae (Wheeler et al. 1990), the centriolar adjunct lies between the nucleus and both mitochondrial derivatives. On the other hand, in Chalcidoidea (Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b) the centriolar adjunct is located laterally to the final portion of the nucleus, surrounding the nuclear-flagellum transition and extending parallel to the axoneme for a short distance, above the insertion of both mitochondrial derivatives. Contrary to the majority of these insects, no centriolar adjunct was encountered in Scelionidae (Lino-Neto and Dolder 2001a). The great variation in shape and location of the centriolar adjunct, differing from that known for most insects (Jamieson 1982) is probably the reason for the earlier misinterpretations of this element in various Hymenoptera (Cruz-Höfling et al. 1970; Quicke et al. 1992).

The mitochondrial derivatives of *P. droryana* are asymmetric not only in length but also in diameter. As a rule, the derivatives are straight (ex. Quicke et al. 1992; Jamieson et al. 1999; Wheeler et al. 1990; Lino-Neto et al. 2000b), but in Chalcidoidea (Lee and Wilkes 1965; Hogge and King 1975; Quicke et al. 1992; Quicke 1997; Lino-Neto et al. 1999, 2000a, 2000b; Lino-Neto and Dolder 2001b), Scelionidae (Lino-Neto and Dolder 2001a) and Diapriidae (Quicke, personal communication) they spiral around the axoneme. The larger mitochondrial derivative beginning next to the final projection of the nucleus was also observed in *A. mellifera* (Lino-Neto et al. 2000b) and in Cynipoidea

(Newman and Quicke 1999b). This is not the case of the majority of the Hymenoptera, where the larger mitochondrial derivative abuts the nuclear base, not overlapping it (ex. Quicke et al. 1992; Wheeler et al. 1990; Jamieson et al. 1999; Newman and Quicke 1999a). In Megalyroidea (Newman and Quicke 2000), Diapriidae (Quicke, personal communication) and Scelionidae (Lino-Neto and Dolder 2001a) the large mitochondrial derivative projects parallel to the nucleus for a considerable distance, and in this latter family, only one large mitochondrion is observed (Lino-Neto and Dolder 2001a). In transverse sections of the *P. droryana* flagellum, four distinct regions make up the larger derivative while only three are found in the smaller one. The same organization was observed in *A. mellifera* (Lino-Neto et al. 2000b) although Cruz-Höfling et al. (1970), Lensky et al. (1979) and Peng et al. (1992, 1993) have described the presence of paracrystalline material also in the smaller derivative. In the Formicidae the mitochondrial derivatives consist in three regions (Wheeler et al. 1990). However, the regions described in Formicidae are not analogous to those in the smaller derivative of *P. droryana*. In Formicidae, there is a clear area, a well developed region of cristae and the paracrystalline material situated in the mitochondrion's first third, proximal to the axoneme (Wheeler et al. 1990). Asymmetrical diameters of mitochondrial derivatives are frequently found, occurring in the Symphyta (Quicke et al. 1992; Newman and Quicke 1999a), Cynipoidea (Quicke et al. 1992; Newman and Quicke 1999b), Megalyroidea (Newman and Quicke 2000) and Proctotrupoidea (Quicke et al. 1992). However, bees are even more strongly asymmetrical (Cruz-Höfling et al. 1970; Hoage and Kessel 1968; Lensky et al. 1979; Peng et al. 1992, 1993; Lino-Neto et al. 2000b). On the other hand, some Hymenoptera have symmetrical mitochondrial derivatives as in Formicidae (Wheeler et al. 1990) and

Chalcidoidea (Lino-Neto et al. 1999, 2000a).

Plebeia droryana, as is common to most insects (Jamieson et al. 1999), has an axoneme with the microtubules arranged parallel to each other. This is not the case of Chalcidoidea (Lee and Wilkes 1965; Hodge and King 1975; Quicke et al. 1992; Quicke 1997; Lino-Neto et al. 1999, 2000a, 2000b; Lino-Neto and Dolder 2001b), Scelionidae (Lino-Neto and Dolder 2001a) and Diapriidae (Quicke, personal communication) where they follow a spiraling course. Also in *P. droryana*, the accessory microtubules are the last ones to terminate at the end of the axoneme. This characteristic is also observed in *A. mellifera* (Peng et al. 1993; Lino-Neto et al. 2000b) and in Formicidae (Wheeler et al. 1990), while in Chalcidoidea (Lino-Neto et al. 1999; Lino-Neto and Dolder 2000a, b) and Ichneumonidae (Braconidae) (Newman and Quicke 1998) the accessory tubules terminate first. Unfortunately, this characteristic has not been taken in consideration by most studies of hymenopteran spermatozoa. We believe this could be a useful parameter to help separate the Aculeata, or parasitic wasps, from other Hymenoptera.

The triangularly shaped accessory bodies, as found in transverse sections of *P. droryana*, are encountered in most Hymenoptera (Quicke et al. 1992; Jamieson et al. 1999; Wheeler et al. 1990; Lino-Neto et al. 2000b). They may be considerably reduced in Chalcidoidea (Lino-Neto et al. 1999, 2000a; Lino-Neto and Dolder 2001b) and in the Scelionidae (Lino-Neto and Dolder 2001a) so that, in some cases, they are difficult to identify. The function of this structure has not been clearly established but they appear to be involved in the attachment of the mitochondrial derivatives on to the axoneme, since they do not occur between the centriolar adjunct and the axoneme.

In *P. droryana* a small central structure was identified between both the mitochondrial derivatives and the axoneme

(see asterisk in Figs. 18 and 19). This structure was initially described in Formicidae (Wheeler et al. 1990), but it is possible that it is present in the majority of Hymenoptera (Lino-Neto et al. 2000b).

Based on the characteristics compared above, the spermatozoa of this bee are, for the most part, similar to the majority of the Hymenoptera (Jamieson et al. 1999). Some distinct differences stand out. For example: (1) the arrangement of spermatozoa in spermatodesmata in the seminal vesicle, (2) the presence of a very long centriolar adjunct between the nucleus and the smaller mitochondrial derivative and (3) the presence of abundant paracrystalline material, exclusively in the large mitochondrial derivative.

The identification of these characteristics and other more subtle ones suggest that the sperm cell can furnish a character matrix for Hymenoptera that will be useful for future phylogenetic studies.

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Structure and Ultrastructure of Spermatozoa in Meliponini (Stingless Bees)
(Hymenoptera: Apidae)

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ABSTRACT

In spite of their importance in the reproduction of phanerozoans, few bees have received an adequate description of their sperm ultrastructure. In this study, we concluded that it was possible to define a characteristic pattern for Meliponini spermatozoa. However, we have also found species-specific differences that could be used, for example, in a taxonomic or phylogenetic evaluation. Meliponini spermatozoa are made up of a head and a flagellar region. The head includes an acrosome containing the *perforatorium*, covered by the acrossomal vesicle and a nucleus. In transverse sections, the acrosome is circular at the tip but becomes triangular as it nears the nucleus. The *perforatorium* base penetrates into a small cavity in the nuclear tip. The flagellum consists of an axoneme, a pair of mitochondrial derivatives, a centriolar adjunct and a pair of accessory bodies. The axoneme has a typical 9+9+2 microtubule pattern. In the final portion, the axoneme becomes gradually disorganized with the accessory microtubules terminating last. The mitochondrial derivatives are asymmetric in length and diameter. In cross section, they are ellipsoidal and the larger one has a paracrystalline region. The centriolar adjunct begins at the nuclear base and extends parallel to the axoneme up to the anterior end of the smaller mitochondrial derivative.

INTRODUCTION

Recent studies consider the subfamily Apinae to consist of 19 tribes (Michener, 2000), including Apini, Bombini, Euglossini and Meliponini, which with the exception of the parasitic genera, make up the group characterized by the presence of specialized basket on the tibiae of the worker females, termed “corbiculae”.

The Meliponini are largely characterized by reduction of the sting. These stingless bees, similar to honey bees (Apini) and unlike Euglossini and Bombini, are highly social, where the female castes, queen and workers, are morphologically and behaviorally very different. They are pantropical, although their greatest abundance and diversity are in the Americas. Currently, 21 genera and several hundreds of species have been recognized (Michener, 1990). The exact number of species cannot be ascertained because of the abundance of cryptic species (Michener, 2000). This group is important in relation to both environmental and economical factors, because their members are the most expressive pollinators, sometimes exclusive for a certain species, thus, contributing to the great diversity of tropical phanerograms, as well as maintaining the species cultivated by man.

Currently, much is known about bee biology, especially social behavior and sex determination (Moure, 1951, 1961; Gauld & Bolton, 1988; Roig-Alsina & Michener, 1993; Gauld & Hanson, 1995; Crozier & Pamilo, 1996; Michener, 1974, 1990, 1994, 2000). However, phylogenetic relationships are still widely debated. Various studies in this area attempted to establish correspondence between morphological, behavioral and molecular character systems. However, these methods have supplied varied hypotheses, many times, contradictory, so that a consensus has not yet been reached (e.g. Chavarría & Carpenter, 1994; Dowton & Austin, 1994; Alexander & Michener, 1995).

Recently, many studies have demonstrated that the structure and the ultrastructure of Hymenopteran spermatozoa, as in other insects (Jamieson et al., 1999), provide additional characters for cladistic analysis and so can contribute to our understanding of relationships (Baccetti, 1972; Dallai, 1979; Quicke et al., 1992; Dallai & Afzelius, 1990, 1995; Carcupino et al., 1995; Jamieson et al., 1999; Lino-Neto et al., 1999, 2000a, b; Lino-Neto & Dolder, 2001a, b, 2002; Zama et. al., 2001). However, there is little information on spermatozoa of this insect order, and to date the spermatozoa of only two bees, *Apis mellifera* (Apini) (Hoage & Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993; Lino-Neto et al., 2000b) and *Plebeia droryana* (Meliponini) (Zama et al., 2001), have been described in detail. Here we describe spermatozoal ultrastructure for 10 species of Meliponini, representing 7 genera, which we hope will be useful in future phylogenetic analyses in Apidae.

MATERIAL AND METHODS

Adult males of *Friesela schrottkyi*, *Frieseomelitta varia*, *Melipona bicolor*, *M. marginata*, *M. quadrifasciata anthidioides*, *M. rufiventris*, *Nannotrigona punctata*, *Scaptotrigona postica* and *Tetragonisca angustula* were obtained from colonies and maintained in the central Apiary of the Federal University of Viçosa, MG, Brazil. Specimens of *Celetrigona longicornis* were obtained from the Bee Laboratory at the University of São Paulo, Campus of Ribeirão Preto, SP, Brazil.

Light Microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where the sperm were spread and fixed in a solution of 4% (wt/vol) paraformaldehyde in

0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60), equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min. with 0.2 µg/ml 4,6-diamino-2-phenylindole (DAPI) in phosphate buffered saline, washed, and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360-370 nm excitation filter.

Transmission Electron Microscopy

Seminal vesicles were dissected and fixed for 3 hours in a solution containing 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.2. The materials were post fixed in 1% osmium tetroxide, in the same buffer, for 1-2 hours. Dehydration was carried out in acetone and embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with the Zeiss LEO 906 transmission electron microscope.

For detection of basic proteins, the ethanolic phosphotungstic acid method (E-PTA), was applied. Seminal vesicles were fixed only in a buffered glutaraldehyde solution for 24 h at 4 °C. After washing in cacodylate buffer and dehydrating in alcohol, the material was treated "en bloc" with a solution of 2% PTA in absolute ethanol for 2h at room temperature and embedded in Epon 812 resin. Some ultrathin sections were observed unstained and part stained with uranyl acetate.

RESULTS

In general, Meliponini sperm structure corresponds to that described for the majority of the Hymenoptera. These cells are long and slender, including a head region with an acrosome and a nucleus and a tail region with an axoneme, a centriolar adjunct, a pair of mitochondrial derivatives and a pair of accessory bodies (Figs. 1-4, 6-7). The total length varies from 300 μm to 80 μm , with the longest sperm being found in *Friesela schrottkyi* (Fig. 1) and *Frieseomelitta varia* and the shortest ones in *Nannotrigona punctata*, *Melipona marginata* and *Scaptotrigona postica* (Fig. 2).

In the seminal vesicle of sexually mature males, the spermatozoa were not regularly arranged. However, they were found still organized in bundles (spermatodesmata), or fragments of these bundles in young adults (Figs. 5-8). In the bundles, the acrosome and anterior nuclear tip are inserted in the relatively loose extracellular matrix (parenthesis in Fig. 6 and asterisks in Figs. 7-8, 14), where the central spermatozoa are ahead of those on the periphery, so as to give different profiles in a single cross section (Figs. 7 & 8).

The acrosome is short, varying between about 1.1 μm in *Tetragonisca angustula* to 1.6 μm in *Melipona bicolor* (Fig. 7). The latter is made up of an acrosomic vesicle and a perforatorium (Figs. 8-15). The acrosomal vesicle forms a cone covering most of the perforatorium (Figs. 8-15). The perforatorium penetrates into a short cavity of the nucleus tip for a distance that varies from 0.1 μm , in *N. punctata*, to 0.3 μm , in *F. schrottkyi* (Figs. 8, 11-12, 14). Between the nuclear tip and the acrosomal vesicle, there is an annular, electron dense, granular structure (Figs. 8 & 14). The anterior extremity of the acrosome is rounded off, covering the tip of the perforatorium (Figs. 12 & 13). However in *S. postica*

and *N. punctata*, the acrosome is projected briefly beyond the perforatorium, forming a narrower tip (Figs. 14 & 15).

In cross section, the acrosome shows a round profile at the tip, which becomes triangular as it nears the nucleus (Figs. 8-10). When circular, an electron lucent layer separates the acrosomic vesicle and the perforatorium, but when triangular this layer is reduced to the vertices (asterisks in Figs. 8-10). Both the acrosomal vesicle and perforatorium are E-PTA negative, with light markings on their surfaces (Figs. 9-11, 13, 15). The granular ring (level 4, in Fig 8 & 14), however, seems lightly positive (arrowhead in Fig. 13).

The nucleus varies in length from approximately 12 μm in *T. angustula* to 7.6 μm in *Melipona bicolor* (respectively, Figs. 3 & 4). Most of the species studied had dense uniform chromatin (Figs. 7-8, 18-21). However, in *Melipona marginata* (Fig. 16) and, more clearly, in *Melipona rufiventris* (Fig. 17), the chromatin is condensed in many aggregates that are not evenly compacted. In transverse sections, the nucleus appears circular, however, in *Celetrigona longicornis*, it presents an oval shape (compare Figs. 19 & 20).

With the E-PTA methodology, the nucleus is observed to be a negative structure. However, cross sections have revealed a crescent shaped area that is strongly E-PTA positive, and which is very evident along most of its length though it tends to disappear in both extremities (Fig. 26). When this material was stained with uranyl acetate, a strongly contrasted region was observed in cross sections coinciding with the E-PTA positive region (curved arrow inset in Fig. 27). In tangential sections, the clear areas could correspond to loose chromatin which was not observed in osmium post fixed material, but occurs consistently in two species (arrows in Fig. 27). The nucleus forms a cone shaped projection

at its posterior end, which is covered by thin, amorphous layers of variable electron density and slightly E-PTA positive (close triangles in Figs. 18, 20-21, 28).

The axoneme presents the typical pattern of $9 + 9 + 2$ microtubules, with electron dense material between the accessory microtubules (Fig. 30). Only the accessory tubules were observed to have E-PTA positive cores (Figs. 26, 33-34). In the centriole, the accessory ones and the doublets can be clearly recognized, separated by an electron dense layer that is E-PTA positive ????? (Figs. 18, 20, 22, 26, 28). In the final region, the two central microtubules finish first, followed by nine doublets and, finally, the accessory microtubules (Figs. 33 & 36).

The centriolar adjunct is compact, very electron dense and long, measuring on the average 5-7 μm in length (Fig. 25). In longitudinal sections, it is rod shaped and approximately circular in cross section (Figs. 20, 22-26, 28). The centriolar adjunct diameter varies among the species (compare Figs. 20, 22-23). The centriolar adjunct usually begins at the nuclear base, extends parallel to the axoneme and the large mitochondrial derivative and finishes just above the anterior tip of the smaller mitochondrial derivative (Figs. 21-22, 25-26, 28). Thus, in cross section, the centriolar adjunct is usually observed next to the centriole, or above it (Figs. 22 & 26). However in *F. schrottkyi*, *F. varia* and *N. punctata*, it is projected anteriorly, where it lies beside the nucleus, and therefore is found, in cross sections, together with the end of the nucleus and the beginning of the large mitochondrial derivative (Fig. 20). In most of the species studied, the centriolar adjunct terminates adjacent to the smaller mitochondrial derivative, where it extends diagonally for a short distance beside the tail structures (Figs. 24 & 25). The centriolar adjunct is strongly E-PTA negative, stained only on the surface (Figs. 26, 28, 34).

The mitochondrial derivatives follow the axoneme along almost all of its length. They differ not only in length but also in diameter (Figs. 29-33), since the large one begins beside the nuclear base (Figs. 18, 20-23) and the smaller, at the posterior end of the centriolar adjunct (Fig. 25). They also terminate unequally, with the smaller one finishing first (Figs. 35 & 36). In cross section, the derivatives are approximately ellipsoidal and the larger one curves somewhat over the smaller (Figs. 29-32).

In most species the larger derivative is about twice the size of the smaller, but in *F. varia*, *T. angustula* and *S. postica*, the areas are not very different for the two derivatives (Fig. 29). The morphology of the derivatives also varies for the different species (see Table 1).

Both derivatives are made up of different areas that can be indicated in cross sections, as follows: a) circular and approximately central area, with low electron density; b) very electron dense and filling in most of the derivative; c) region of the cristae (letters a, b, and c in the Fig. 32). The cristae are limited to a region opposite to the axoneme (Figs. 16, 18, 32, 33). The larger mitochondrial derivative also possesses a paracrystalline region (p in Figs. 20, 26, 30, 32-34) in its distal third. Occasionally, in *M. quadrifasciata* and *F. varia*, paracrystalline material was observed also in the smaller derivative, although much reduced. Both derivatives are E-PTA negative, with exception of the paracrystalline region (Figs. 26, 33-34).

The accessory bodies are long structures placed laterally between the axoneme and mitochondrial derivatives. In cross sections they have a triangular profile (stars in Figs. 29-32, 35). Near the centriolar adjunct, only one accessory body can be found between the axoneme and the single mitochondrial derivative observed here (Figs. 20 & 22). The

second accessory body appears only together with the smaller mitochondrial derivative, below the centriolar adjunct. These bodies are completely E-PTA negative.

DISCUSSION

Meliponini spermatozoa, in the seminal vesicle, are generally free and scattered at random, as observed in the other Apocrita (Quicke et al., 1992; Newman & Quicke, 1998, 1999b; Jamieson et al., 1999; Lino-Neto et al., 1999; Lino-Neto et al., 2000a, b; Lino-Neto & Dolder, 2001a, b, 2002). However, we found some spermatozoa still forming discrete spermatodesmata or attached to remnants of this structure. Spermatodesmata have previously been described in Symphyta, which are considered the most basal Hymenoptera, (Quicke et al., 1992; Newman & Quicke, 1999a) and in the Meliponini, *Plebeia droryana* (Zama et al., 2001). Structurally, the bundles of other Meliponini are similar to those described for *P. droryana* (Zama et al., 2001), where the central spermatozoa are somewhat ahead of the peripheral ones, making a rounded bundle apice. Although Zama et al. (2001) described spermatodesmata as typical of *P. droryana*, we now believe that the presence of this arrangement is common to all the Meliponini, or maybe even all Aculeata in early stages of adult development, and that they may be lost as the adults mature sexually. Therefore, the observation of fragments of this structure in the seminal vesicle would reflect the remains of the testicular sperm organization. The fact that spermatozoa in the seminal vesicle are organized in spermatodesmata in Symphyta, temporarily in Aculeata and not in parasitic wasps (non Aculeata), suggests that either a reversion has occurred in Aculeata or this group is phylogenetically closer to Symphyta than the parasitic wasps (see Table 2).

The structure of Meliponini spermatozoa is similar to the general description for insect sperm (Phillips, 1970; Baccetti, 1972). They are linear, as in the majority of the Hymenoptera. However, they differ from Chalcidoidea and Scelionidae in that they are not spirally twisted (see Table 2).

The acrosome is formed only by the acrossomal vesicle and perforatorium (double layered pattern) as was also observed in *P. droryana* (Zama et al., 2001) and *Melipona bicolor bicolor* (Cruz-Landim, 2001, Fig. 6), and also in the, *Apis mellifera* (Cruz- Höfling et al., 1970; Hoage & Kessel, 1968; Lensky et al., 1979; Peng et al., 1992, 1993), in the Scelionidae, *Trissolcus basalis* (Lino-Neto & Dolder, 2000a), in Formicidae (Wheeler et al., 1990; Lino-Neto & Dolder, 2002) and "Symphyta", *Tremex* sp. (Newman & Quicke, 1999a). This structure is similar in all bees, although, in some Meliponini, the acrosomic vesicle is curved anteriorly, just above the perforatorium tip or forms a narrow anterior projection (Table 1). This projection also occurs in *Apis mellifera* (Hoage & Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993), although the narrow portion is clearly longer (about 1 μm) than was observed in the Meliponini. As also occurs in the majority of the Hymenoptera, the perforatorium base fits into a small cavity of the nuclear tip (Quicke et al., 1992; Newman & Quicke, 1999a; Wheeler et al., 1990; Lino-Neto & Dolder, 2002). Furthermore, in transverse sections, the acrossomal vesicle of Meliponini, appears circular in cross sections of the tip, tending toward a triangular profile as it reaches the nucleus (Zama et al., 2001; Cruz-Landim, 2001, fig. 6), In Euglossini (Zama et al., *in preparation*) and Bombini (*personal observation*), this structure is also cylindrical at the anterior tip but tends to become oval, in cross section, for most of its length, leaving a conspicuous lateral space between itself and the circular perforatorium.

However, in *Apis mellifera* both the acrossomal vesicle and perforatorium have an ellipsoidal cross sections for their entire length (Hoage & Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993). In other Hymenoptera, the acrosome always remains circular in cross section (e. g. Symphyta, Newman & Quicke, 1999a; Cynipoidea, Newman & Quicke, 1999b; Chalcidoidea, Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b, and Formicidae, Wheeler et al., 1990; Lino-Neto & Dolder, 2002). Unlike most of the Apocrita, the acrosomic vesicle, the perforatorium and the nuclear tip are encapsulated by an extracellular layer or "extracellular sheath", that may even be associated with a series of filaments, as found in Chalcidoidea (Table 2). In Meliponini, not only the acrosomic vesicle, but also the perforatorium are E-PTA negative, as was also observed in the parasitic wasps, *Trichogramma pretiosum* and *T. atopovirilia* (Lino-Neto et al., 2000a). However in the Eurytomidae, *Bephratelloides pomorum*, the acrosome was lightly positive (Lino-Neto et al., 1999).

The loosely arranged chromatin of *M. marginata* and *M. rufiventris* spermatozoa is very surprising and differs from other Hymenoptera, including other Meliponini. Lino-Neto and Dolder (2002) state that the nucleus of *Solenopsis invicta* has many electron clear areas, but this is not the same characteristic.

The basal nuclear projection in Meliponini is not as evident as in *A. mellifera*, where the nucleus narrows to fit into the centriole (Table 1). In the other Hymenoptera, the nucleus ends abruptly (Quicke et al., 1992; Newman & Quicke, 1999a, b; Wheeler et al., 1990; Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b). The nucleus of spermatozoa, treated with E-PTA, is homogeneous and completely negative in other Hymenoptera (Lino-Neto et al., 1999, 2000a, b) as in Lepidoptera (França & Báó, 2000; Mancini, 2002, personal communication) and Coleoptera (Báó, 1991, 1996, 1998; Báó &

Hamú, 1993). Therefore, it was surprising to find a positive crescent moon shaped area in the nucleus in Meliponini, when viewed in cross section. This area varies in size along the length of the nucleus and may represent a concentration of basic proteins. Since this portion of the chromatin is not evident with other methods, it is ambiguous to affirm that this is due to differential chromatin condensation. On the other hand, uranyl acetate staining of this material showed another clear area in *N. punctata* and *S. postica*, interpreted as loose chromatin or alterations due to the lack of osmium post fixation. E-PTA staining has not been carried out on many Hymenoptera, so it is hard to generalize about results obtained with this method.

In Meliponini, the centriolar adjunct is linear, with a regular diameter, agreeing with the description for *P. droryana* (Zama et al., 2001), while in *A. mellifera*, the diameter increases so that it is initially very narrow (Lino-Neto et al., 2000b) (Table 1). In the typical arrangement for Meliponini, the centriolar adjunct appears in an asymmetrical pattern as occurs in Symphyta, except in the Siricoidea *Tremex* sp. (Newman & Quicke, 1999a), Cynipoidea (Newman & Quicke, 1999b), Ichneumonoidae (Quicke et al., 1992) and in *A. mellifera* (Lino-Neto et al., 2000b). Although in Ichneumonoidea this structure is comparatively short (Quicke, 1997), in *A. mellifera*, it is extremely long (Lino-Neto et al., 2000b). On the other hand, the centriolar adjunct may be placed between the nucleus and the two mitochondrial derivatives (symmetric pattern) as is found in the "Symphyta", genus *Tremex* sp. (Newman & Quicke, 1999a) and in the Formicidae (Wheeler et al., 1990; Lino-Neto & Dolder, 2002). In Chalcidoidea, the centriolar adjunct is located in front of both derivatives and it projects anteriorly, beside the nucleus, surrounding the transition area from nucleus to flagellum (Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b).

The Scelionidae present another extreme because they appear to completely lack a centriolar adjunct (Lino-Neto & Dolder, 2001a) (see Table 2).

The larger mitochondrial derivative beginning beside the nuclear projection, as described for other bees (Lino-Neto et al., 1999; Zama et al., 2001), is a distinguishing character. In other Hymenoptera, the large derivative begins below the nuclear base and not parallel to it (e.g. Quicke et al., 1992; Wheeler et al., 1990; Jamieson et al., 1999; Newman & Quicke, 1999a; Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b). However, in some parasitic wasps one of the mitochondrial derivatives begins just above the nuclear base (e.g. in the Cynipoidea *Leptopilina heterotoma*, Newman & Quicke, 1999b), extending along almost all the nucleus (e.g. in the Megalyroidea *Megalyra fasciatipennis*, Newman & Quicke, 2000) or even projecting beyond the nucleus (e.g. in Scelionidae, Lino-Neto & Dolder, 2001a). Also, in Scelionidae, only one mitochondrial derivative was found (Lino-Neto & Dolder, 2001a). Asymmetrical diameters of the two derivatives, as described for Meliponini, are typical for the Hymenoptera (Table 2). In contrast, other bees and the sawfly, *Xyela julii* have a very extreme asymmetry, while in the Formicidae, this difference is more subtle (Wheeler et al., 1990; Lino-Neto & Dolder, 2002). Finally, in the sawfly, *Tremex* sp. (Newman & Quicke, 1999a) and in Chalcidoidea (Lino-Neto et al., 1999, 2000a) the mitochondrial derivatives are equal in diameter.

A detailed description of the various regions in the derivatives has been made for *A. mellifera* (Lino-Neto et al., 2000b) and *P. droryana* (Zama et al., 2001). However, in some Meliponini, paracrystalline material was also found in the smaller derivative, but this is a rare and very small area. Members of the Formicidae possess a considerable amount of paracrystalline material in both derivatives, located in the anterior thirds (Wheeler et al., 1990; Lino-Neto & Dolder, 2002).

The axoneme has the same structure as occurs in other Hymenoptera. However, the order of microtubule termination, described by few authors, can be an important difference. In Meliponini (Zama et al., 2001), Apini (Peng et al., 1993; Lino-Neto et al., 2000b), Euglossini (Zama et al., *in preparation*) and Bombini (*personal observation*) the accessory microtubules are the last ones to end at the flagellum tip. In Formicidae, the microtubules end practically together (Wheeler et al., 1990; Lino-Neto & Dolder, 2002). On the other hand, in Chalcidoidea (Lino-Neto et al., 1999; Lino-Neto & Dolder, 2000a, 2001b) and Ichneumonoidea (Newman & Quicke, 1998), the accessory microtubules are the first to terminate. Thus, this characteristic is consistent and may possibly be used to differentiate between Aculeata and other Apocrita (Table 2).

In conclusion, the recognition of these characteristics and a few more subtle ones suggest that hymenopteran spermatozoa may furnish a valid character matrix for future phylogenetic studies.

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TABLE 1

Groups	Acrosomal vesicle	Chromatin	Nuclear E-PTA positive structure	Nuclear posterior tip	Centriolar adjunct X Nucleus	Centriolar adjunct	Mitochondrial derivatives	References
Meliponini								
<i>F. schrottkyi</i>	Concave	Condensed chromatin	Present	Projected eccentric	Slightly overlap the nuclear tip	Rod shape; Posterior tip eccentrically projected	Elongated	
<i>F. varia</i>	Concave	Condensed chromatin	Present	Projected eccentric	Slightly overlap the nuclear tip	Rod shape; Posterior tip eccentrically projected	Slightly elongated	
<i>M. bicolor</i> *	Slightly projected	Condensed chromatin	?	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Slightly pear shape	
<i>M. marginata</i>	Concave	Slightly loose chromatin	Present	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Pear shape	
<i>M. quadrfasciata</i>	Concave	Condensed chromatin	Present	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Oval	
<i>M. rufiventris</i>	Concave	Loose chromatin	?	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; ?	Oval	
<i>N. punctata</i>	Projected	Condensed chromatin	Present	Projected eccentric	Slightly overlap the nuclear tip	Rod shape; Posterior tip eccentrically projected	Oval	
<i>S. postica</i>	Projected	Condensed chromatin	Present	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Slightly elongated	
<i>T. angustula</i>	Concave	Condensed chromatin	?	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Elongated	
<i>C. longicornis</i>	Concave	Condensed chromatin	?	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; ?	Slightly elongated	* also in Fig 6, Cruz-Landim, 2001
<i>P. droryana</i>	Concave	Condensed chromatin	?	Projected eccentric	Begin posterior to the nuclear tip	Rod shape; Posterior tip eccentrically projected	Oval	Zama et al, 2001
Apini								
<i>Apis mellifera</i>	Markedly projected	Condensed chromatin	?	The posterior tip inserted in the axonemal arrangement	Slightly overlap the nuclear tip	Cone shape; Finish abrupt	Almost circular	Cruz-Höfling et al, 1970; Lensky et al, 1979; Peng et al., 1992, 1993; Lino-Neto et al., 2000b

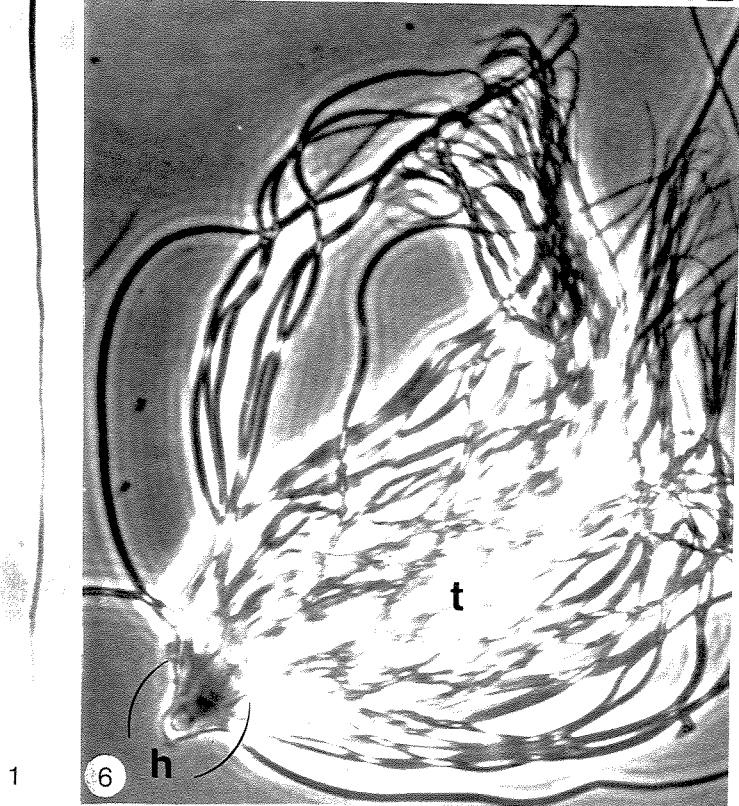
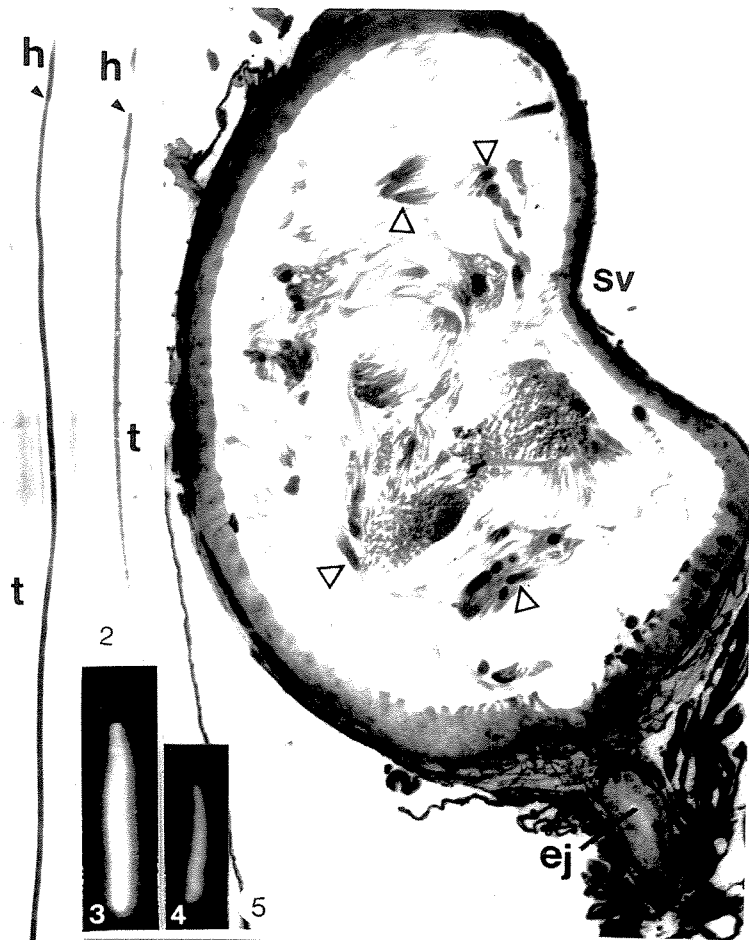
TABLE 2

Groups	Acrosome	Axoneme (posterior tip)	Centriolar adjunct	Mitochondrial derivatives	Sperm	References
Symphyla	Generally, 2 layers	?	Asymmetric, except in Siricoidea	Asymmetric, except in Siricoidea	Linear; spermatodesmata	Quicke et al., 1992; Newman & Quicke, 1999a.
Apocrita Apoidea						
Apidae	2 layers	The doublets and two central microtubules finish first.	Asymmetric	Asymmetric in diameter; Paracrystalline material in only one derivative	Linear; No spermatodesmata	Cruz-Höfling et al. 1970; Lensky et al. 1979; Peng et al., 1992, 1993; Lino- Neto et al., 2000b
Vespoidea						
Formicidae	2 layers	The microtubules ends simultaneously	Symmetric	Slightly asymmetric in diameter; Paracrystalline material in both derivatives	Linear; No spermatodesmata	Thompson & Blum, 1967; Wheeler et al., 1990; Lino-Neto & Dolder, 2002.
Chalcidoidea						
Eulophidae	3 layers	?	?	?	Spiral; No spermatodesmata	Wilkes & Lee, 1965.
Pteromalidae	3 layers	?	?	Symmetric in diameter	Spiral; No spermatodesmata	Hogge & King, 1975.
Eurytomidae	3 layers	The accessory microtubules finish first.	Symmetric, projected anteriorly beside the nucleus, surrounding nucleus-flagellum transition.	Symmetric in diameter	Spiral; No spermatodesmata	Quicke et al., 1992; Lino-Neto et al., 1999.
Trichogrammatidae	3 layers	The accessory microtubules finish first.	Symmetric, projected anteriorly beside the nucleus, surrounding nucleus-flagellum transition.	Symmetric in diameter	Spiral; No spermatodesmata	Lino-Neto et al., 2000a; Lino-Neto & Dolder, 2001b.
Ichneumonoidea						
Braconidae	2 layers	The accessory microtubules finish first.	Asymmetric	Symmetric in diameter	Linear; No spermatodesmata	Newman & Quicke, 1998.
Platygaстроidea						
Scelionidae	2 layers or absent	?	Absent	Only one mitochondrial derivative; Presents paracrystalline material	Spiral; No spermatodesmata	Lino-Neto & Dolder, 2001a.
Cynipoidea						
Eucillidae	3 layers	?	Asymmetric	Asymmetric in diameter; Paracrystalline material in only one derivative	Linear; No spermatodesmata	Newman & Quicke, 1999b.
Megalyroidea						
Megalyridae	2 layers	?	Asymmetric	Asymmetric in diameter; Paracrystalline material in only one derivative	Linear; No spermatodesmata	Newman & Quicke, 2000.

FIGURE LEGENDS

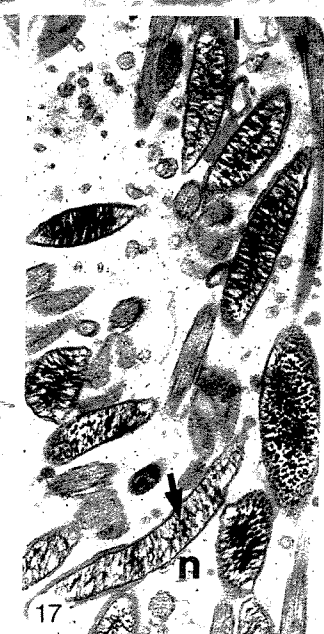
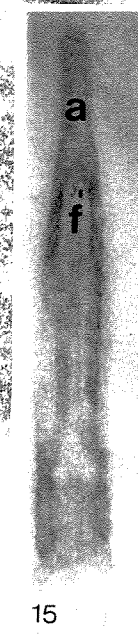
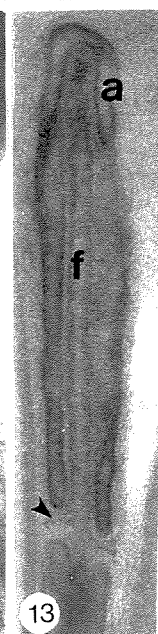
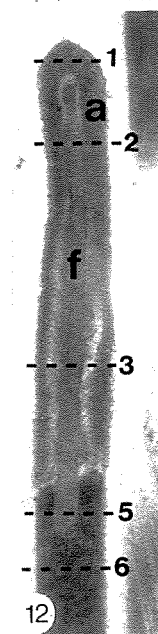
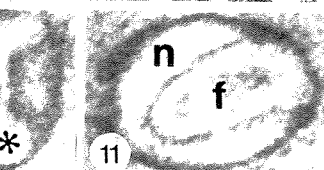
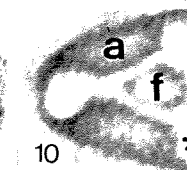
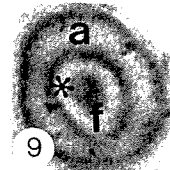
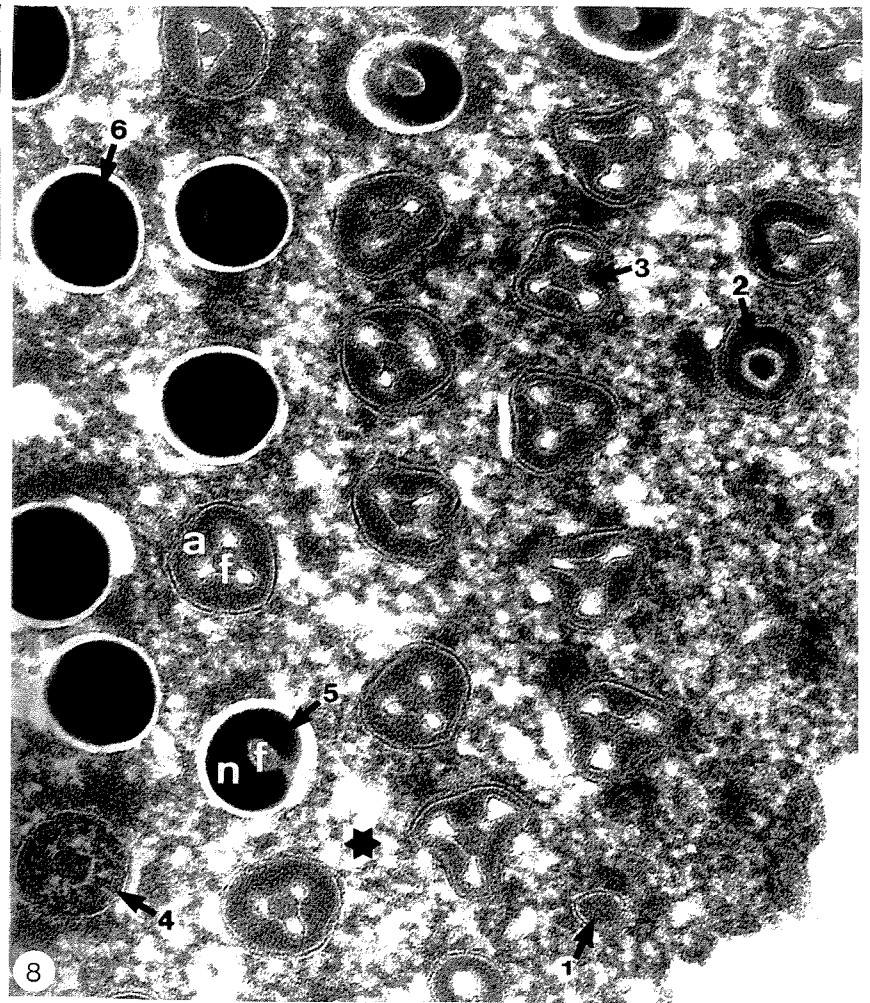
Figs. 1-6 Light Microscopy of Meliponini spermatozoa

- 1-2. Spermatozoa of *Friesela schrottkyi* and *Scaptotrigona postica*, respectively, observed with phase contrast microcopy. The arrowheads indicate the limit between head (h) and tail (t). x 272.
- 3-4. Spermatozon nucleus of *Tetragonisca angustula* and *Melipona bicolor*, respectively, marked with DAPI fluorescence. x 2,000.
- 5-6. Aspect of the spermatodesmata bundles observed in the seminal vesicle (sv) and isolated from this organ in *Nanotrigona punctata*. 5. The open triangle indicates the anterior portion of the bundles and (ej), ejaculatory duct. 6. The open parenthesis indicates the (h) head and (t) tail regions . 5: x 290; 6: x 455.



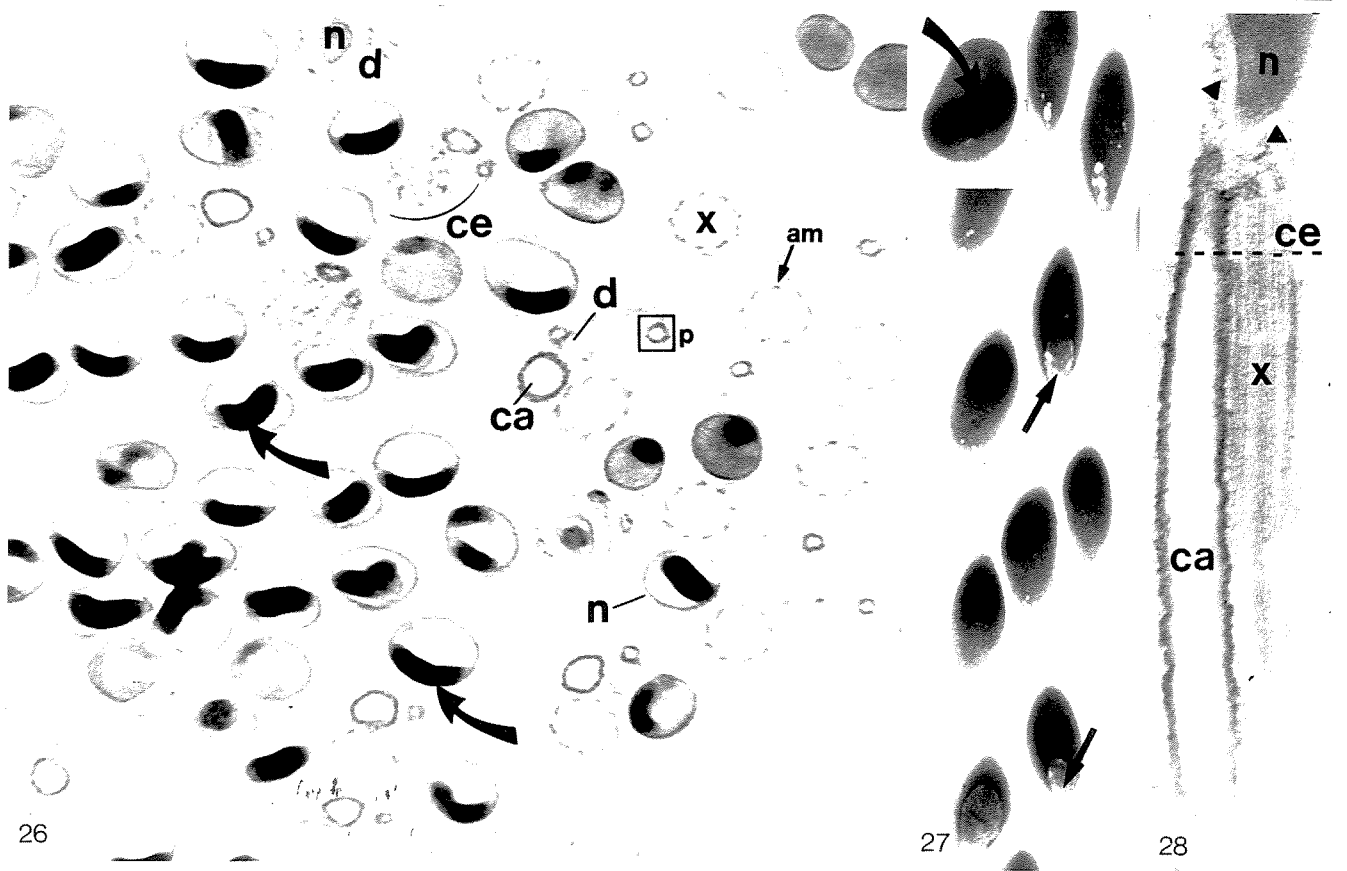
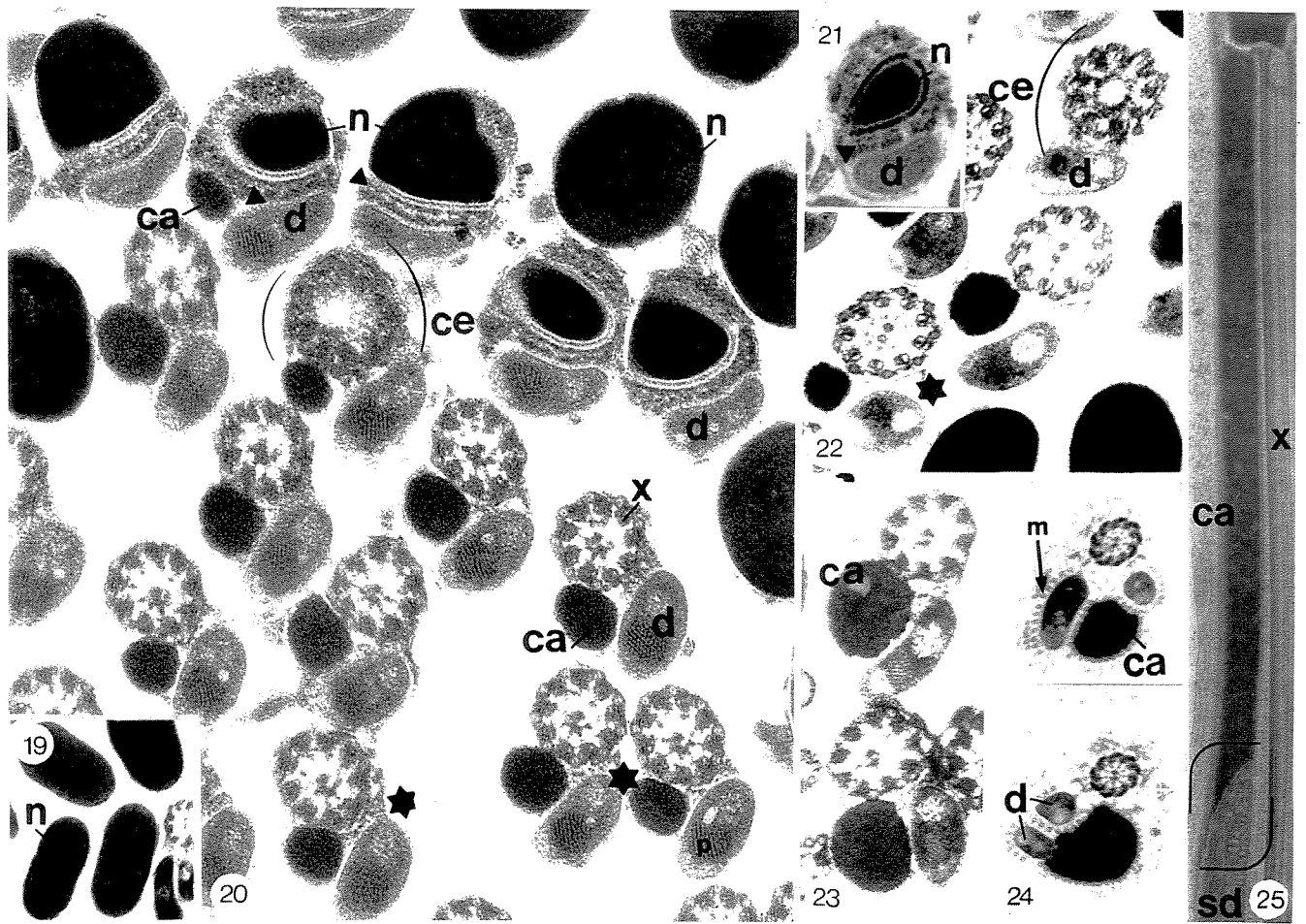
Figs. 7-18. MET of head region in the seminal vesicle

7. Longitudinal section of spermatodesmata bundle of *N. punctata*. (ac) acrosomes; (n) nucleus; (★) loose extracellular matrix of the spermatodesmata (also in Figs. 8 and 14.); (h) head and (t) tail. x 10,000.
- 8-11. Cross sections of acrosomal region of *N. punctata* (8) and *M. marginata* (9-11, E-PTA). The numerical sequence on Fig. 8 indicates transverse sections of 1) the acrosomic vesicle, 2) circular profile of acrosome and perforatorium, 3) triangular acrosome and perforatorium, 4) granular ring, 5) nucleus penetrated by the perforatorium and 6) the nucleus. The asterisk indicates the electron-lucent and E-PTA negative region between acrosomal vesicle and perforatorium. (a) acrosomal vesicle; (f) perforatorium. 8: x 79,000. 9-11: x 156,000.
- 12-15. Longitudinal sections of the acrosome *Melipona quadrifasciata* (12), *M. marginata* (13, E-PTA) and *N. punctata* (14, conventional fixation, 15, E-PTA). The numbered broken lines show the levels sectioned transversally in figures 8; the arrowhead shows the slightly E-PTA positive granular ring. (a) acrosomal vesicle; (f) perforatorium. 12: x 48,000; 13, 15: x 63,000; 14: 51,000.
- 16-18. Longitudinal and transverse section of nuclei of *M. marginata* (16) and *M. rufiventris* (17) showing loose chromatin and a longitudinal section of the nuclear-flagellum transition region of *M. quadrifasciata* (18) showing typical condensed chromatin. (►) indicates the nuclear projection surrounded by layers of loose electron dense material; (▷) mitochondrial cristae; (d) mitochondrial derivative; (x) axoneme; (n) nucleus. The broken line corresponds to the centriolar region (ce) as in Figs. 20, 22, 26 and 28. 16: x 46,000; 17: x 13,000; 18: x 33,000.



Figs. 19-28 MET of anterior portion of flagellum

19. Cross section of oval nucleus in *Celete trigona longicornis*. x 22,000.
- 20-23. Transverse section of the nuclear-flagellum transition in *N. punctata* (20), *M. quadrifasciata* (21), *M. bicolor* (22) and *Frieseomelitta varia* (23). At this level, the centriolar adjunct (ca) appears with variable diameters or may be absent. (►) indicates the nuclear projection surrounded by layers of loose electron dense material; (★) accessory bodies; (n) nucleus; (d) mitochondrial derivative; (x) axoneme; (ce) centriolar region; (p) paracrystalline material. 20: x 62,000; 21: 52,000; 22: 47,000; 23: 54,000.
- 24-25. Cross sections of the contact region of centriolar adjunct and the smaller mitochondrial derivative (sd) in *F. schrottkyi* spermatids (24) and spermatozoa (25). In 25, the centriolar adjunct ends on the diagonal above the smaller mitochondrial derivative. (m) microtubular manchette. 24: x 26,4000; 25: x 18,000.
26. Transverse section of *M. marginata* flagellum with E-PTA. Observe crescent shaped E-PTA positive region of the nucleus (n, curved arrow). Only the accessory microtubules (am) and intertubular connections are positive for E-PTA in the axoneme (x, ce), while E-PTA negative structures include the mitochondrial derivatives (d), except for the paracrystalline region (p), the accessory bodies and the centriolar adjunct (ca) (only stained on the surface). x 37,000.
27. Tangential and transverse sections of nuclei post fixed with E-PTA and uranyl acetate stained in *N. punctata* and *Scaptotrigona postica* (inset), respectively. Arrows indicate an electron-lucent nuclear region and the curved arrow indicates the E-PTA positive structure. x 22,000.
28. Longitudinal section of *M. marginata* flagellum with E-PTA. x 48,000.

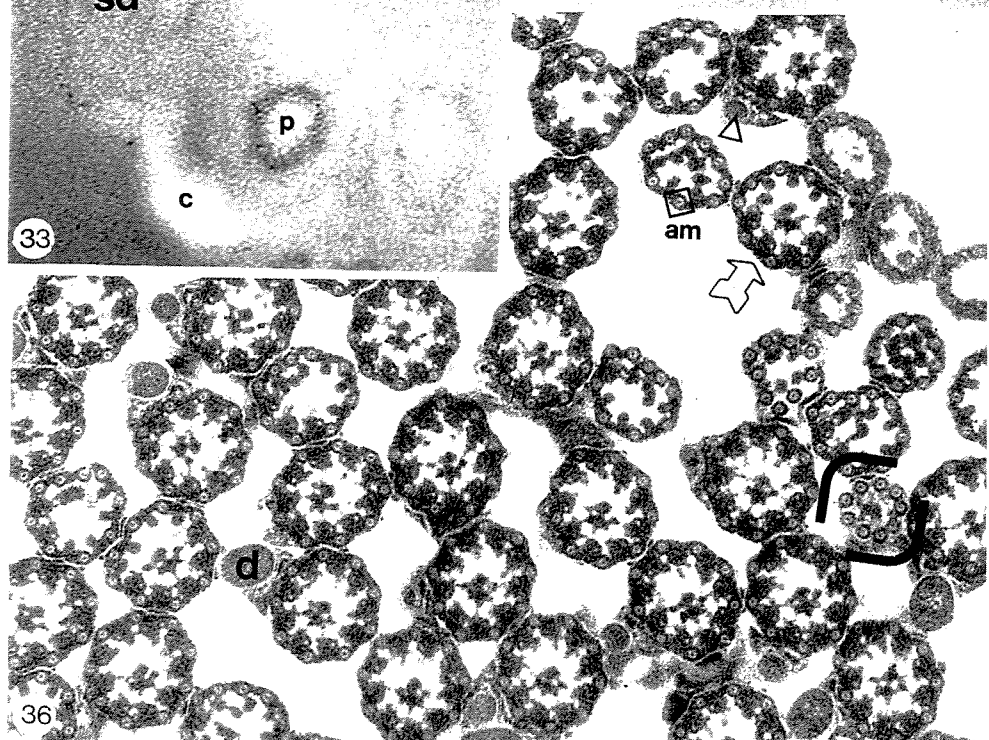
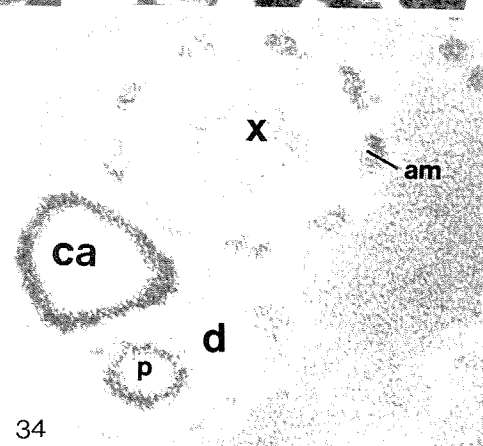
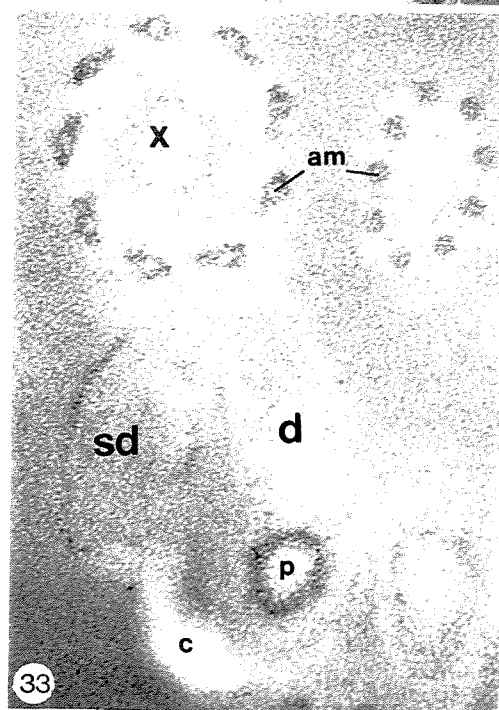
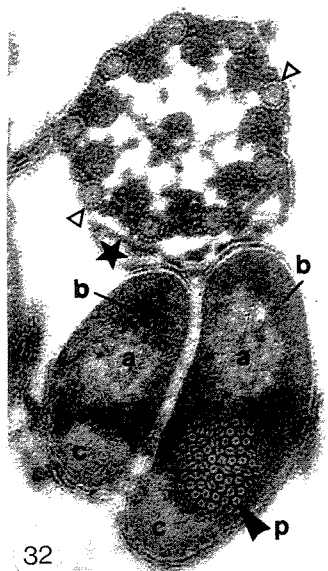
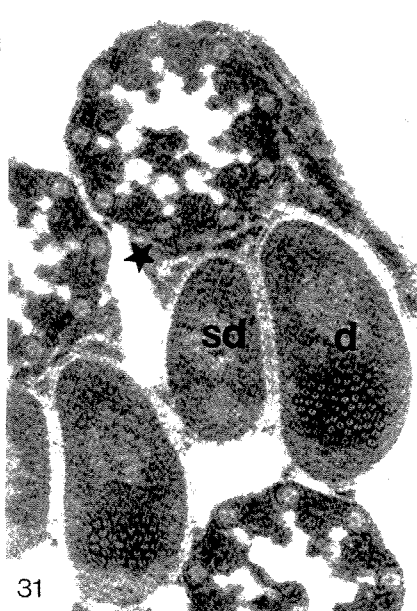
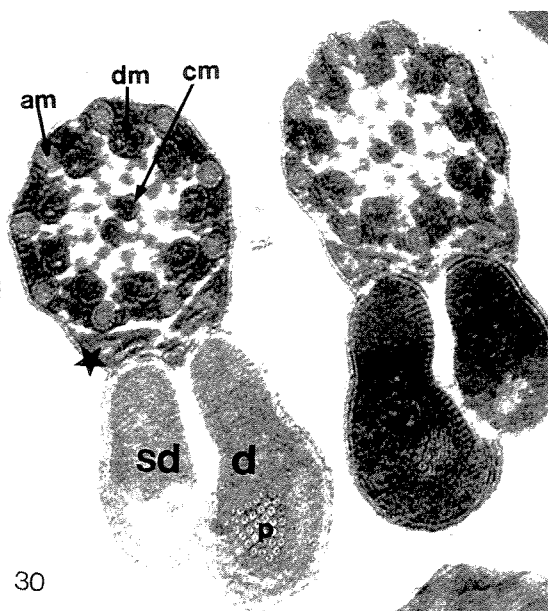
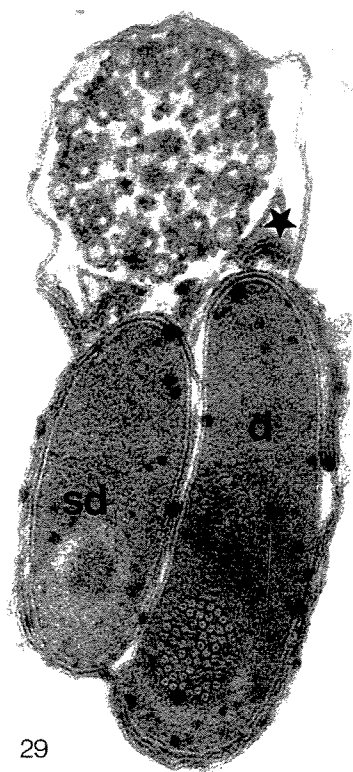


Figs 29-36

29-32. Transverse sections of a flagellum of *Tetragonisca angustula* (29), *M. marginata* (30), *N. punctata* (31) and *M. bicolor* (32). The axonemes are made up of 9 accessory microtubules (am), nine doublets (dm) and a central pair (cm) (Fig. 30). In Fig. 32, the mitochondrial derivatives are divided in regions: (a) is appoximately central and of low electron density, (b) fills in most of the derivative and is dense, (c) cristae region and (p) paracrystalline material found in the larger derivative. (★) accessory bodies; (▷) protofilaments of microtubules; (sd, d) smaller and larger mitochondrial derivatives. x 136,000.

33-34. Transverse sections of *M. marginata* flagellum with E-PTA. x 155,000.

35-36. Cross sections of flagellum tips of *M. marginata* and *N. punctatata*, respectively. The larger mitochondrial derivative extends further than the smaller one (Fig. 36, ▷). Notice in Fig. 36, the sequence of axoneme disorganization, where the doublets and central microtubules terminate first and the accessory ones, later (detail). These accessory microtubules stand out with E-PTA, in Fig. 33. Arrow indicate the axonemal tip without derivatives. (d) larger mitochondrial derivative. 35: x 124,000; 36: x 72,000.



2. TRIBO EUGLOSSINI

- **MANUSCRITO 2:**

Em fase final de preparação para submissão à publicação no periódico "Insectes Sociaux"

Zama, U., Lino-Neto, J., Melo, S.M. & Dolder, H. **Ultrastructural characterization of spermatozoa in Euglossini bees (Hymenoptera: Apidae: Apinae).**

Ultrastructural characterization of spermatozoa in Euglossini bees (Hymenoptera: Apidae: Apinae)

Running head: Ultrastructure of Euglossini spermatozoa

Keywords: Ultrastructure; spermatozoa; Euglossini; Apidae; Hymenoptera

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This is an original submission

SUMMARY

The Euglossini spermatozoa are the longest described for Hymenoptera. This cell includes a head and a flagellar region. The head consists in an acrosome, which is made up of the acrossomal vesicle, covering the perforatorium, and a nucleus. In transverse sections, the acrosome is circular at the tip but appears oval along most of its length. The perforatorium base penetrates into a deep cavity in the nuclear tip. The flagellum consists in an axoneme, a pair of mitochondrial derivatives, a centriolar adjunct and a pair of accessory bodies. The axoneme has a 9+9+2 microtubule pattern. In the final portion, the axoneme is gradually disorganized, with the central microtubules and the nine doublets terminating simultaneously, followed by the accessory microtubules. The mitochondrial derivatives are asymmetric both in length and diameter. Sectioned transversally, the derivatives are ellipsoidal or have a pear shape. The larger one has the more expressive paracrystalline region. The centriolar adjunct begins at the nuclear base and extends parallel to the axoneme until it encounters the smaller mitochondrial derivative, on which it fits, making a concave groove. In this study, we obtained a pattern typical for Euglossini spermatozoa, however we also have species-specific differences that could be used, for example, in a taxonomic or in a phylogenetic evaluation.

INTRODUCTION

The Euglossini are one of 19 tribes that constitute the Apinae subfamily (Michener, 2000). This tribe, together with the Apini, Bombini and Meliponini, make up a group characterized by the presence of corbiculae on the third tibia of the female worker with exception of the parasitic genera.

The Euglossini males collect aromatic chemicals to produce essences attractive to bees of the opposite sex. With this complex and unusual behavior, they pollinate, along with other species, the majority of the orchid species of tropical regions, which justifies their generic denomination of "orchid bees" (Hanson and Gauld, 1995; Michener, 2000).

The Euglossini tribe includes five genera, *Euglossa*, *Eulaema*, *Eufriesea*, *Exaerete* and *Aglae* (Michener, 2000). They are uncommonly large bees, about 8.5 mm to 29 mm (Michener, 2000), and very colorful, differing from other corbiculated bees by their frequently metallic hues, while others are very hairy and dark (Kimsey, 1987; Hanson and Gauld, 1995). Other important characteristic is a long proboscis (long tongues), that reach at least to the base of the metasoma (Hanson and Gauld, 1995; Silveira et al., 2002).

The Euglossini also differ significantly from other corbiculated Apidae in that they are not an eusocial group. Some species of *Eufriesea* and *Euglossa* are solitary, however, some *Eufriesea* often produce aggregations of cells in protected places, constructed by several, or many, females (Michener, 2000). There are also some genera, such as *Euglossa* and probably all *Eulaema*, that are organized in parassocial colonies (Michener, 2000; Silveira et al., 2002). The genera *Aglae* and *Exaerete* are cleptoparasitic to the nests of *Eulaema* and *Eufriesea* (Michener, 2000).

The cladistic relationships between Euglossini and other corbiculated tribes are not clear. Phylogenetic studies based on somatic, behavioral and molecular characters suggest

different hypotheses, sometimes contradictory (Wille, 1979; Kimsey, 1984; Ranistsyn, 1988; Camargo and Wittmann, 1989; Prentice, 1991; Sheppard and McPherson, 1991; Cameron, 1991, 1993; Cameron et al., 1992; Camargo and Pedro, 1992a, b; Chavarría and Carpenter, 1994; Dowton and Austin, 1994; Alexander and Michener, 1995; Pignata and Diniz-Filho, 1996; Roubik et al., 1997; Dowton et al., 1997; Mardulyn and Cameron, 1999; Ronquist et al., 1999; Koulianos and Schmid-Hempel, 2000; Noll, 2002).

Various recent studies have demonstrated that the structure and the ultrastructure of spermatozoa in Hymenoptera, as in other insects (Baccetti, 1987; Jamieson et al., 1999), furnish sufficient variations to permit the construction of a consistent character matrix that could be applied to cladistic analysis. This could contribute to the confirmation of phylogenetic relationships (Baccetti, 1972; Dallai, 1979; Quicke et al., 1992; Dallai and Afzelius, 1990, 1995; Carcupino et al., 1995; Jamieson et al., 1999; Lino-Neto et al., 1999, 2000a, b; Lino-Neto and Dolder, 2001a, b, 2002; Zama, 2001). However, there are few reports on the spermatozoa of this insect order. In corbiculated bees, there is a detailed description of only one species of Apini, *A. mellifera* (Hoage and Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993; Lino-Neto et al., 1999) and some Meliponini (Zama et al., 2001, 2003 no prelo). In this study, we describe the spermatozoa of six species representing four Euglossini genera, with the intention of contributing toward future phylogenetic studies in these corbiculated bees, as well as to this type of research in Apoidea as a whole.

MATERIAL AND METHODS

Adult males of *Euglossa cordata* Linnaeus (1758), *Euglossa mandibularis* Friese (1899), *Eulaema cingulata* Fabricius (1804), *Eulaema nigrita* Lepeletier (1841), *Exaerete smaragdina* Guérin (1845), *Eufriesea violacea* Blanchard (1840), were collected on the campus of the Federal University of Viçosa and the surrounding city of Viçosa, MG, Brazil. The bees were collected, with entomological nets and traps containing the aromatic substances: vanilin, cineole, eugenol and benzil acetate.

Light Microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where the sperm were spread and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60), equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min. with 0.2 µg/ml 4,6-diamino-2-phenylindole (DAPI) in phosphate buffered saline, washed and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360-370 nm excitation filter.

Transmission Electron Microscopy

Seminal vesicles were dissected and fixed for 3 hours in a solution containing 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.2. The materials were post fixed in 1% osmium tetroxide, in the same buffer, for 1-2 hours. Dehydration was carried out in acetone and embedding in Epon. Ultrathin sections

were stained with uranyl acetate and lead citrate and observed with the Zeiss LEO 906 transmission electron microscope.

For basic protein detection, the ethanolic phosphotungstic acid method (E-PTA), was applied. Seminal vesicles were fixed only in buffered glutaraldehyde solution for 24 h at 4 °C. After washing in cacodylate buffer and dehydrating in alcohol, the material was treated "en bloc" with solution of 2% PTA in absolute ethanol for 2h at room temperature and embedded in Epon 812 resin.

RESULTS

The Euglossini spermatozoa are linear, thin and exceptionally long. Their length varies from 720 μm , in *Eulaema nigrata* (Fig. 1), to about 1500 μm , in *Euglossa mandibularis*. In the seminal vesicle, the sperm are dispersed at random, although bundle fragments were found in some cases (Fig. 6).

The spermatozoa include a head and a tail region (limited by arrowhead on Fig. 1). The head is made up of an acrosome and a nucleus, while the tail, or flagellum, includes an axoneme, a centriolar adjunct, a pair of mitochondrial derivatives and a pair of accessory bodies.

In the species studied here, the acrosome varies in length between 2,5 to 3 μm and consists in an acrosomal vesicle and the perforatorium. The acrosomal vesicle is cone shaped and covers the perforatorium up to the beginning of the nucleus (Figs. 2-3). The perforatorium base is inserted in a cavity in the asymmetric anterior tip of the nucleus (Figs. 2-3). This cavity measures approximately 0.63 μm in depth in *Eufriesea violacea* (Fig. 3).

Transection of the acrosomic vesicle shows an oval profile which becomes more circular as the section nears the tip (Figs. 4-5). When circular, a regular electron lucent layer separates the acrosomic vesicle and the perforatorium, but when it is oval, this layer is thinner at the smaller diameter (Figs. 4-5). The axonemal vesicle is E-PTA positive, while the perforatorium and the layer between them are negative (Fig. 5).

The nucleus is rather long, measuring about 46 μm in *Eulaema nigrata* and 55 μm in *Euglossa mandibularis*. It is slightly oval in cross section (Figs. 4-7) with very dense chromatin (Figs. 3-6, 9-11). The anterior nuclear tip is very asymmetric (Fig. 3), so that it is possible to section transversally the perforatorium together with a portion of the nucleus

and of the acrosomal vesicle (level 3 on Figs. 7, 22). The nucleus is E-PTA-positive, with medium density, and depending on the sectioning level, a small area, restricted to the center, may be intensely stained (Figs. 5, 7). The posterior extremity is funneled down eccentrically so that the tips of the centriolar adjunct and the larger mitochondrial derivative can fit in beside the nucleus base (Figs. 9-11).

The axoneme presents the $9 + 9 + 2$ pattern, where the nine single accessory microtubules are the most external, followed by the nine doublets and a central pair (Figs. 12, 15). Only the accessory tubules and some intertubular material were E-PTA positive (Figs. 20, 22). In the terminal region, the central microtubules and the nine doublets finish first, followed by the accessory microtubules (Figs. 23, 27).

In these species, the mitochondrial derivatives differ in length and diameter, with the larger one beginning first, adjacent to the nuclear extremity (Figs. 9-11), and extending beyond the smaller derivative in the flagellar tip (Figs. 23-26).

Cross sections of the mitochondrial derivatives of *Eulaema cingulata*, *Eulaema nigrita* (Figs. 15, 20, 22), *Eufriesea violacea* (Fig. 18-19, 24) and *Exaerete smaragdina* the mitochondrial derivatives are ellipsoidal and the larger one has twice the area of the smaller one. In *Euglossa mandibularis* (Fig. 16-17) e *Euglossa cordata* (Figs. 8, 12, 23), the larger derivative is markedly pear-shaped and has at least three times the area of the smaller one.

In all species, the mitochondrial derivatives are divided into the regions: (a) a clear, circular, centrally located region, (b) an electron dense region adjacent to the axoneme that fills in around the other structures, (c) the mitochondrial cristae region, which is restricted to the border that is distal in relation to the axoneme, (p) the paracrystalline region, occurring only in the larger derivative (Figs. 8, 15-16, 18-20, 22). This latter region (p) is strongly E-PTA negative (Figs. 20, 22). Longitudinal sections of the cristae show them to

be perpendicular to the long axis of the derivatives and spaced at regular intervals that measure 39 nm in *Euglossa mandibularis* (Fig. 13) and 44 nm in *Eulaema nigrita*.

The centriolar adjunct is long, situated below the nucleus and above the smaller mitochondrial derivative, so that it remains parallel to the other flagellar organelles (asymmetric pattern) (Figs. 8, 10-12, 14-15, 17, 19-21). Transections of the centriolar adjunct are approximately triangular shape (Figs. 8, 12, 17, 19-20) and, depending on the level sectioned, this structure may attain a diameter equal to the larger mitochondrial derivative at its side (Figs. 12, 15, 19-20). The terminal portion of the adjunct slants outward so that the anterior tip of the smaller mitochondrial derivative fits in, against the axoneme (Fig. 8). The adjunct is compact and electron dense (Figs. 8, 10-12, 14-15, 17, 19). However, in *Eulaema nigrita*, it presents various clear areas (Figs. 14-15). After E-PTA treatment, this structure is not electron dense, with the exception of its outer surface (Figs. 20-21).

The accessory bodies are approximately triangular, in cross section. They are located between the axoneme and only the mitochondrial derivatives and, therefore, are not seen accompanying the centriolar adjunct (Figs. 8, 12). A small structure, approximately cylindrical and extending along most of the flagellum, is regularly found between the two mitochondrial where they contact the axoneme (Fig. 15-16, 18, 26).

DISCUSSION

Spermatozoa in Euglossini, measuring 720 μm to 1500 μm , are the longest that have been described in the Hymenoptera order. According to Quicke (1992), the shortest spermatozoa in this order are the braconid wasps (e.g. *Meteorus* sp., measuring 8 μm , *Macrocentrus* sp. with 9 μm and *Blacus* sp., *Aliolus* sp., and *Microplitis* sp., with 12 μm). The longest sperm previously reported were those of the aculetan *Eumenes fraternus* (Eumeninae), with 577 μm (Quicke, 1992) and the parasitic wasp *Bephratelloides pomorum* (Eurytomidae), which reaches 620 μm (Lino-Neto et al., 1999). In Apidae, the length of the sperm of some species of Andrenidae (432 μm), Anthophoridae (218 μm), Megachilidae (221 μm e 266 μm), and Xylocopinae (207 μm) has been reported (Quicke et al., 1992). Considering the four corbiculated tribes of Apinae, the spermatozoan lengths were registered for Apini (*Apis mellifera*, with 255 μm by Cruz-Höfling et al. 1970) and Bombini (*Bombus terrestris* with 168 μm , *B. pascuorum* with 181 μm and *B. lapidarius* with 230 μm by Quicke et al., 1992). In Meliponini, they vary from 80 μm (in *Nannotrigona punctata*) to 300 μm (in *Frieseomelitta varia*) (Zama et al., 2003, no prelo).

The spermatozoa of Euglossini have many ultrastructural characteristics in common with other Hymenoptera (Baccetti, 1972; Wheeler et al., 1990; Quicke et al., 1992; Newman and Quicke, 1998, 1999a, b, 2000; Jamieson et al., 1999; Lino-Neto et al., 1999, 2000a, b; Lino-Neto and Dolder, 2001a, b, 2002, Zama et al., 2001). They have a long nucleus, an acrosome made up of an acrosomal vesicle and a perforatorium (bilayered), an axoneme in the typical 9 + 9 + 2 microtubule pattern, two mitochondrial derivatives and a centriolar adjunct. However, there are some peculiarities for this tribe.

The bilayered acrosome are frequently found in Hymenoptera. It has been described in Symphyta (Quicke et al., 1992; Newman and Quicke, 1999a), in the parasitic wasp *Trissolcus basalis* (Lino-Neto and Dolder, 2001a), in ants (Wheeler et al., 1990; Lino-Neto and Dolder, 2002) and in bees (Hoage and Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al. 1992, 1993; Zama et al., 2001, 2003 no prelo). However, in many parasitic wasps the acrosome presents a third layer, an extracellular sheath, which covers all the acrosomic vesicle and part of the nucleus (Quicke et al., 1992; Newman and Quicke, 1998, 1999b; Lino-Neto et al., 1999, 2000a; Lino-Neto and Dolder, 2001a). On the other hand, in the Scelionidae, *Telenomus podisi*, no structure similar to an acrosome was observed (Lino-Neto and Dolder, 2001a).

Although the bilayered acrosome is also found in Apinae, some differences could be observed between the three corcubilated tribes previously described. In *A. mellifera*, a representative of the Apini, the acrosome is very long, with approximately 5,6 μm in length (Lensky et al., 1979; Peng et al., 1992, 1993) and has a thin anterior projection measuring about 1 μm in length (Lensky et al., 1979). Also there is a dense structure between the acrosomal vesicle and the tip of the perforatorium (Hoage and Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993). In Euglossini, the anterior tip of the acrosomal vesicle thins regularly over a short distance. However, in Meliponini (Zama et al., 2001, 2003, no prelo), the tip is rounded terminating abruptly covering the perforatorium tip, as was observed in some Symphyta (Newman and Quicke, 1999a) and Formicidae (Wheeler et al., 1990, Lino-Neto and Dolder, 2002). Furthermore, other differences can be found in cross section. The anterior portion of the acrosome is circular in Euglossini, Bombini (personal observation) and Meliponini (Zama et al., 2001, 2003 no

prelo). However, in Euglossini and Bombini cross sections observed along approximately two thirds of the acrosomal vesicle appear oval, while in Meliponini this portion is triangular in shape where it nears the nucleus (Zama et al., 2001, 2003 no prelo). In Apini, not only the acrosomal vesicle but also the perforatorium are strongly ellipsoidal along all of their length (Hoage and Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al. 1992, 1993). In the transition region, the nucleus and the acrosomic vesicle are asymmetrical and complementary in Euglossini (see Fig. 3), as well as in Apini (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993). While in Meliponini, these structures are regular and symmetric at this junction with the nucleus (Zama et al., 2001, 2003 no prelo).

The morphological aspects of the nuclear region, like the cavity in the anterior tip, where the perforatorium base fits in, and the posterior nuclear projection are very similar in all known bees (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993; Lino-Neto et al., 2000b; Zama et al., 2001, 2003 no prelo). The cavity in the anterior nuclear tip differs only in that it is more profound in Euglossini than in *A. mellifera* (Peng et al., 1992, 1993) and Meliponini (Zama et al. 2001, 2003 no prelo).

In *A. mellifera*, the posterior nuclear projection penetrates into the centriole (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al. 1992, 1993, Lino-Neto et al., 2000b), while in Euglossini and Meliponini, the nuclear base finishes above the axonemal implantation. The nucleus of spermatozoa treated with E-PTA is homogeneous in various Hymenoptera (Lino-Neto et al., 1999, 2000a, b), in Lepidoptera (França and Báó, 2000; Mancini, 2002, *personal communication*) and Coleoptera (Báó, 1991, 1996; Báó and Hamú, 1993). However, in Euglossini, as in Meliponini (Zama et al., 2003 no prelo), in the major of sections a long central portion stains strongly positive. This area may represent a

high concentration of basic proteins. Since this nuclear area is not observed with other methods, it is ambiguous to affirm that this is due to differential chromatin condensation.

The centriolar adjunct in Euglossini located between the nucleus and only one mitochondrial derivative, called the *asymmetric pattern*, also occurs in Symphyta, with exception of Siricoidea (Newman and Quicke, 1999a), in Ichneumonoidea wasps (Newman and Quicke, 1998), in Cynipoidea (Newman and Quicke, 1999b), in Apini (Lino-Neto et al., 2000b) and Meliponini (Zama et al., 2001, 2003 no prelo). However, in the symphytan *Tremex* sp. (Newman and Quicke, 1999a) and in Formicidae (Wheeler et al., 1990; Lino-Neto and Dolder, 2002), the centriolar adjunct is located between the nucleus and both mitochondrial derivatives, in a *symmetric pattern*. Furthermore, in Chalcidoidea (Lino-Neto et al., 1999; Lino-Neto et al., 2000a; Lino-Neto and Dolder, 2001b), this structure forms a ring or a semi-circle that surrounds the nuclear base and the anterior extremities of both the mitochondrial derivatives and the axoneme, and may emit a projection, sometimes over a long distance and twisting in a spiral around the nucleus. Also, in Scelionidae, no centriolar adjunct was observed (Lino-Neto and Dolder, 2001a). In Euglossini, in the Meliponini *Nannotrigona puctata* (Zama et al., 2003 no prelo) and in Apini (Hoage and Kessel, 1968) the anterior tip of the centriolar adjunct is projected shortly forward, covering the nuclear base. The posterior end of the centriolar adjunct is approximately concave, covering the smaller mitochondrial derivative tip, in these Euglossini. In Meliponini the adjunct's extremity extends, becoming narrower as it moves away from the axoneme, so that, in cross section, this projection can be found to one side or between the two mitochondrial derivatives (Zama et al., 2001, 2003 no prelo). This transition region has been described in *A. mellifera*, as fitting abruptly onto the axoneme and mitochondrial derivatives (Lino-Neto et al., 2000b). In Euglossini, depending on the cross section level it is possible to find part

of the centriolar adjunct reaching beyond the large mitochondrial derivative with a progressive narrowing at this end. In the Apini, *A. mellifera*, the centriolar adjunct is a rod that has a reduced anterior diameter (Lino-Neto et al., 2000b) while in Meliponini, this structure has a uniform diameter (Zama et al., 2001, 2003 no prelo). Also, in *Eulaema nigrita*, the centriolar adjunct has many electron lucent areas, which has not been observed in other Hymenoptera. The E-PTA negative response, with strong marking on the surface, as described for Euglossini, was also observed for Chalcidoidea (Lino-Neto et al., 1999, 2000a) and in Meliponini (Zama et al., 2003 no prelo). However, in *A. mellifera* this structure is E-PTA positive (Lino-Neto et al., 2000b).

The two mitochondrial derivatives in Euglossini are asymmetric in length, diameter and morphology. The asymmetry in diameter of these structures is common for bees (*A. mellifera*, Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993; Lino-Neto et al., 2000b; Meliponini, Zama et al., 2001, 2003 no prelo; *Halictes* sp. and *Nomada* sp., Quicke et al., 1992), as also for some Symphyta (Newman and Quicke, 1999a), Proctotrupoidea (Quicke et al., 1992), Dryinidae (Quicke et al., 1992), Eucoilidae (Newman and Quicke, 1999b) and Megalyroidea (Newman and Quicke, 2000). However, these derivatives have an approximately equal diameter in Siricoidea (Symphyta, Newman and Quicke, 1999a), Ichneumonoidea (Quicke et al., 1992), Chalcidoidea (Quicke et al., 1992; Lino-Neto et al., 1999; 2000a; Lino-Neto and Dolder, 2001b) and Formicidae (Wheeler et al., 1990; Lino-Neto and Dolder, 2002). In Scelionidae only one mitochondrial derivative was observed (Lino-Neto and Dolder, 2001a).

Furthermore, in bees, the larger mitochondrial derivative presents four different regions while only three are found in the smaller one, when observed a cross section (Lino-Neto et al., 2000b; Zama et al., 2001, 2003 no prelo). These regions can be distinguished on

figures 15 and 16 by the letters **a**, **b**, **c** and **p**. The (**a**) region in Apini, occupies most the smaller mitochondrial derivative (Lino-Neto et al., 2000b), while in Euglossini and Meliponini it is reduced (Zama et al., 2001, 2003 no prelo). In Euglossini and Meliponini (Zama et al., 2001, 2003 no prelo), the cristae (**c**) are located in a narrow peripheral band, opposite to the axoneme. However in Apini, a longer, semi-circular (**c**) region follows the external border of the derivatives (Lino-Neto et al., 2000b).

The (**p**) region, formed by a paracrystalline material, is present exclusively in the larger mitochondrial derivative and occurs in the distal third in relation to the axoneme in bees (Lino-Neto et al., 2000b; Zama et al., 2001, 2003 no prelo). This region is exceptionally large in diameter in Euglossini, where the paracrystal occupies more than a half of this derivative for the *Euglossa* genus and at least one third of the derivative in *Eulaema*, *Eufriesea* and *Exaerete*. In Euglossini, this paracrystalline region is considerably larger than in Apini (Lino-Neto et al., 2000b), Meliponini (Zama et al., 2001, 2003 no prelo) and in the other Hymenoptera in which it has been observed (Wheeler et al., 1990; Newman and Quicke, 1999a, b; Newman and Quicke, 2000; Lino-Neto and Dolder, 2002). However, in Formicidae, the paracrystalline material is present in the both mitochondrial derivatives and it is located next to the axoneme (Wheeler et al., 1990; Lino-Neto and Dolder, 2000). The E-PTA staining varies in bees, where Euglossini and Apini, have negative **a** and **p** regions, while the **b** is positive (Lino-Neto et al., 2000b). However, these derivatives are completely negative, with a positive reaction restrict to the border of the paracrystalline material in Meliponini (Zama et al., 2003 no prelo) and in Coleoptera (Báo, 1991, 1996), while this structure is homogeneously positive in Chalcidoidea (Lino-Neto et al., 2000a) and in Lepidoptera (França and Báo, 2000).

Although the microtubular arrangement pattern is largely conserved in Hymenoptera (Baccetti, 1972; Jamieson et al., 1999) differences can be recognized in the sequence of their cutoff in the final axonemal portion. In all aculeate, the central microtubules and the nine doublets terminate first, followed by the accessory microtubules (bees; Lino-Neto et al., 2000b; Zama et al., 2001, 2003 no prelo) or all terminate approximately together (Formicidae; Wheeler et al., 1990; Lino-Neto and Dolder, 2000). A different order was found for parasitic wasps where the nine doublets are the last microtubules in the flagellum's tip (Lino-Neto et al., 1999, 2000a; Lino-Neto and Dolder, 2001b). Probably, this characteristic indicates a phylogenetic relationship, but other groups should be studied to confirm this suggestion. Furthermore, in bees, only the accessory microtubules and some intertubular material are E-PTA positive (Lino-Neto et al., 2000b; Zama et al., 2003 no prelo). However, in Eurytomidae (Lino-Neto et al., 1999), the axoneme is completely E-PTA positive, while in Trichogrammatidae, all microtubules are negative and the axoneme is covered by an E-PTA-positive material (Lino-Neto et al., 2000a). In other insects, the axoneme is E-PTA positive (Lepidoptera, França and Bão, 2000; Coleoptera, Bão, 1991, 1996).

Accessory bodies are present in the majority of Hymenoptera (Hoage and Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993; Wheeler et al., 1990; Newman and Quicke, 1998, 1999a, b, 2000; Lino-Neto et al., 1999, 2000a, b; Lino-Neto and Dolder, 2001a, b, 2002; Zama et al., 2001, 2003 no prelo). In all cases, these structures are present beside the flagellum, laterally to the mitochondrial derivatives. They are similar, but may vary in size. This structure is E-PTA positive in Euglossini and Meliponini (Zama et al., 2003 no prelo), while it is partially positive in Apini (Lino-Neto et

al., 2000b). In the order Coleoptera, this accessory body is completely E-PTA positive (Báo, 1991, 1996).

Finally, The four Euglossini genera analyzed in this study share various structural and ultrastructural characteristics which permit the establishment of a pattern for the tribe. However, in some aspects, the mitochondrial derivative in *Euglossa* is very different from that in *Eulaema*, *Eufriesea* e *Exaerete*. This may be an indication of a large phylogenetic difference between the genera of *Euglossa* and the other three genera. This hypothesis corroborate Michener (2000), where the most accepted phylogenetic proposal is (*Euglossa* (*Exaerete* (*Eufriesea* + *Eulaema* + *Aglae*))). However, various studies still discuss the position of these genera (see Kimsey, 1982, 1987; Michener, 1990; Engel, 1999; Oliveira, 2000; Silveira et al., 2002).

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FIGURE LEGENDS

Figure 1. Phase contrast of spermatozoa of *Eulaema nigrita*. The arrowhead marks the transition of head (h) and tail (t) regions. Scale bar: μm

Figures 2-5. Acrossome of *Eufriesea violacea* in longitudinal (2, 3) and transversal (4) sections. In 5, transversal sections of *E. nigrita* prepared by E-PTA methodology. The numerated broken lines in figures 2 and 3, shows the levels sectioned transversally in figure 4 and 5: acrossomal vesicle (v) and *perforatorium* (p) with circular (1) and oval (2) shapes; (3) *perforatorium*, seminal vesicle and nucleus (n); (4) *perforatorium* in the nuclear cavity; (5) nucleus. In 3, see the asymmetric nuclear tip, which corresponds to the number 3 on the figures 4 and 5. Scale bar: (2, 4, 5) 154 nm, (3) 133 nm.

Figure 6. Spermatozoa in the seminal vesicle of *Euglossa mandibularis*, shows the fragments of spermatodesmata bundles (bf) at the nucleus (n) level. Scale bar: 1,1 μm .

Figure 7. Transversal section of *E. nigrita* nucleus (n) with E-PTA methodology. See the central region markedly positive (arrows). (t) tail sectioned. Scale bar: 316 nm.

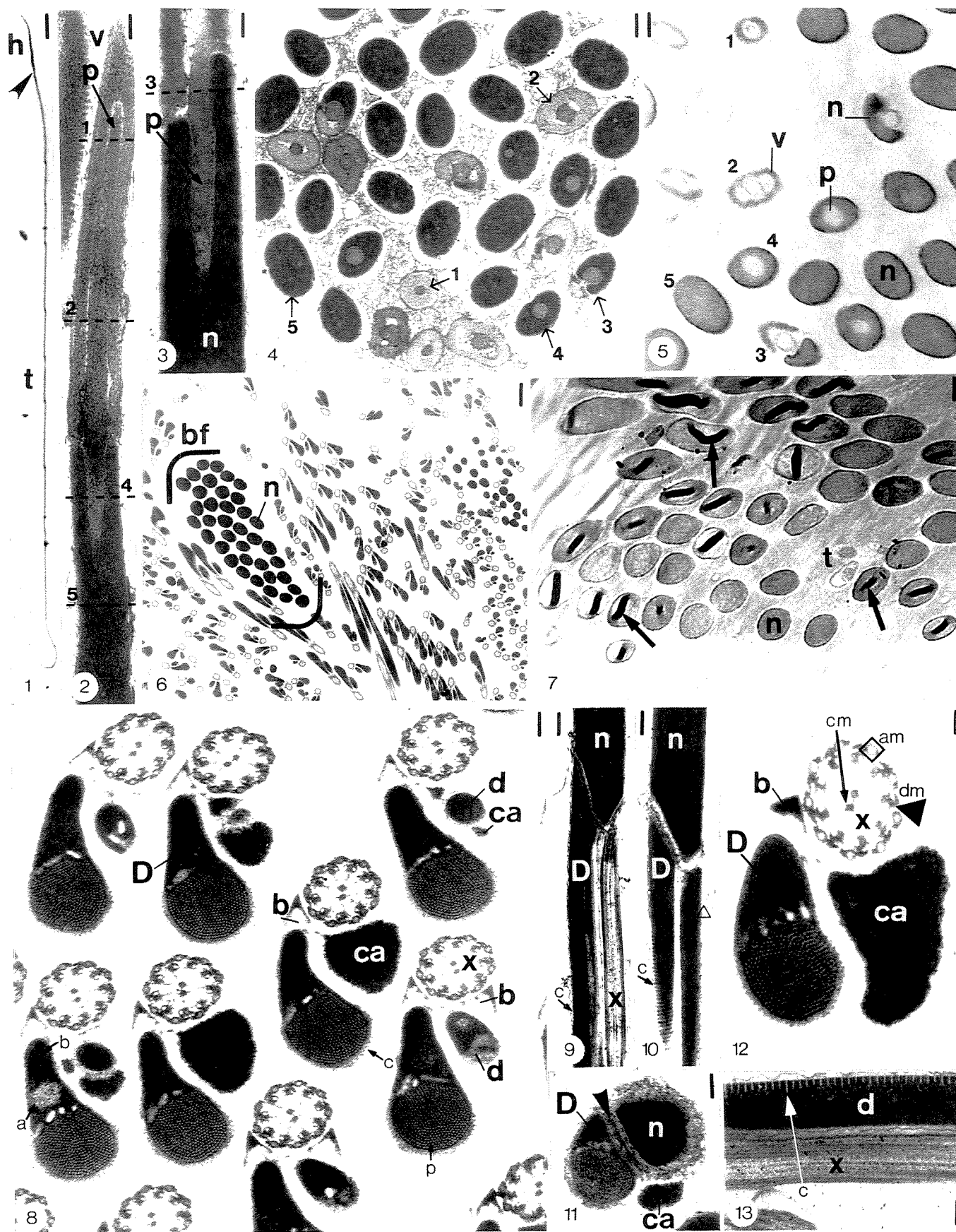
Figure 8. Transversal sections of the anterior region of the *Euglossa cordata* flagellum. See the interface region of centriolar adjunct (ca) and smaller mitochondrial derivative (d). (D) larger mitochondrial derivative, (b) accessory bodies, (x) axoneme, (c) cristae and (p) paracrystalline regions. Scale bar: 88 nm.

Figures 9-10. Longitudinal sections of *Eufriesea violacea* (9) and *E. mandibularis* (10) spermatozoa. Figure 9 shows the nuclear projection and insertion of axoneme and larger mitochondrial derivative, while the figure 10 show the insertion of centriolar adjunct (triangle) in the same region. (c) mitochondrial cristae. Scale bar: 322 nm.

Figure 11. Transversal section of the nucleus-flagellum transition region, show the centriolar adjunct projected parallel to the nucleus and larger mitochondrial derivative. (Arrowhead) dense material around nuclear projection. Scale bar: 96 nm.

Figure 12. Transversal section of *E. cordata* spermatozoa. This figure shows a prominent centriolar adjunct in the anterior portion of flagellum. The axoneme is made up of 9 accessory microtubules (am), nine doublets (triangle - dm) and a central pair (arrow - cm). Scale bar: 63 nm.

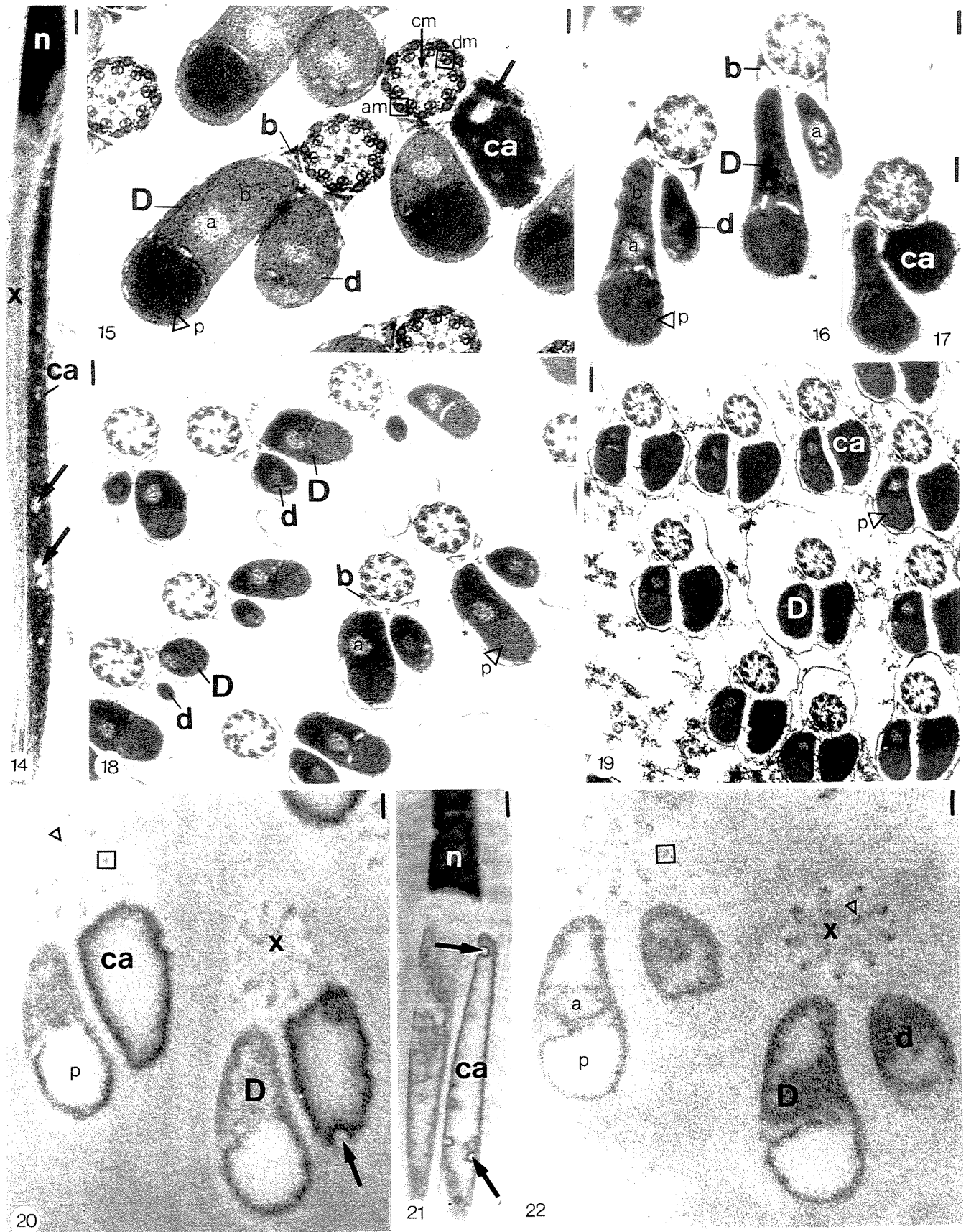
Figures 13. See the regular distribution of mitochondrial cristae (arrow - c) on the mitochondrial derivative (d). Scale bar: 159 nm.



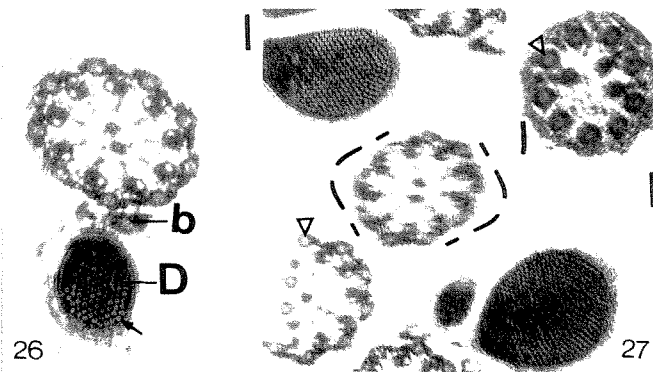
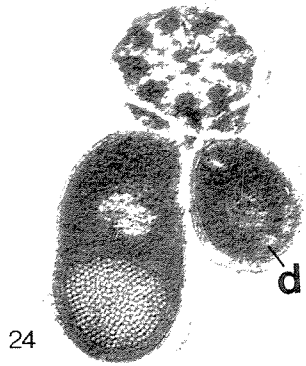
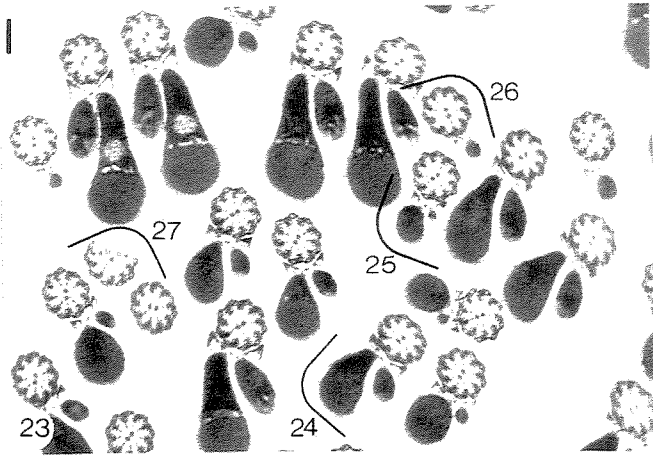
Figures 14-15. Transversal (14) and longitudinal (15) sections of flagellum in *E. nigrita*. This figures shows the inclusions electron-lucent in centriolar adjunct (arrows). (n) nucleus, (x) axoneme, (ca) centriolar adjunct, (b) accessory body, (D, d) bigger and smaller mitochondrial derivative, (p) paracrystalline material, (am, dm, cm) accessory, doublets and central microtubules. Scale bar: (14) 312 nm, (15) 75 nm.

Figure 16-19. Flagellum of *Euglossa mandibularis* (16, 17) and *E. violacea* (18, 19). Observe the asymmetry of the derivatives and morphologic variations between genera. See regions **a**, **b** and **p**. Scale bar: (16, 17) 121 nm, (18) 130 nm, (19) 166 nm.

Figure 20-22. Transversal (20, 22) and longitudinal (21) sections of *E. nigrita* flagellum with E-PTA methodology. See the "holes" in the E-PTA negative centriolar adjunct (arrows). Notes that only the accessory microtubules (am) and intertubular material are positive in axoneme. The **b** and **p** regions are negative, while the **a** one are positive. Scale bar: (20, 22) 66 nm, (21) 200 nm.



Figures 23-27. Final portion of flagellum in *E. cordata* (23, 25-27) and *E. violacea* (24). The larger mitochondrial derivative (D) extends further than the smaller one (d). Note in this figures, the presence of paracrystalline material in the end of larger derivative (arrow). Notice the sequence of axoneme disorganization, where the doublets and central microtubules terminate first and the accessory ones later (triangle). Scale bar: (23) 208nm, (24) 77 nm, (25, 26, 27) 65 nm, (inset) 55 nm.



3. TRIBO BOMBINI

- MANUSCRITO 3:

Em preparação para ser submetido à publicação no periódico “Zoomorphology”

Zama, U., Lino-Neto, J. & Dolder, H. **Sperm structure and ultrastructure of the bumblebee *Bombus (Fervidobombus) atratus* Franklin (Hymenoptera: Apidae: Bombini).**

Sperm structure and ultrastructure of the bumblebee *Bombus (Fervidobombus) atratus* (Franklin) (Hymenoptera: Apidae: Bombini).

Authors:

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Running head: Ultrastructure of Bombini spermatozoa

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RESUMO

Neste trabalho descrevemos pela primeira vez a morfologia e citoquímica de E-PTA dos espermatozóides em um representante da Tribo Bombini. Os espermatozóides de *Bombus (Fervidobombus) atratus* medem cerca de 190 μm e são constituídos pela cabeça e flagelo. A cabeça é constituída pelo acrossomo formado pelo perforatorium e vesícula acrossomal e pelo núcleo. O flagelo é constituído por um axonema de padrão 9+9+2 microtúbulos, um longo adjunto do centríolo, dois derivados mitocondriais assimétricos e dois corpos acessórios. A vesícula acrossomal, o adjunto do centríolo, os corpos acessórios, alguns microtúbulos do axonema e algumas regiões dos derivados mitocondriais são E-PTA positivos. Além destas estruturas, uma região alongada e central do núcleo também é intensamente E-PTA positiva. Esta região também é observada nas tribos filogeneticamente próximas, Meliponini e Euglossini, entretanto seu significado representa uma incógnita.

INTRODUÇÃO

Os Bombini representam uma das dezenove tribos constituintes da subfamília Apinae (Michener 2000). Estes, juntamente com os Apini, Euglossini e Meliponini, constituem as tribos corbiculadas dos Apinae, caracterizadas pela presença de um aparato para coleta de pólen nas tíbias posteriores das operárias, denominado corbícula (Michener 2000).

Atualmente, a tribo Bombini é considerada constituída apenas pelo gênero *Bombus* Latreile, subdivididos em 37 subgêneros, com cerca de 250 espécies (Michener 2000). A maior abundância e diversidade da tribo, ocorre nas regiões de clima frio. No Brasil, são encontradas apenas seis espécies, todas incluídas no subgênero *Fervidobombus* Skorikov, sendo elas *atratus* Franklin, 1913; *bellicosus* Smith, 1879, *brasiliensis* Lepeletier, 1836; *brevivillus* Franklin, 1913; *morio* Swederus, 1787 e *transversalis* Olivier, 1789 (Silveira et al. 2002).

Os Bombini são reconhecidos pela aparência robusta, compreendendo abelhas peludas e escuras, de médio a grande porte, com cerca de 9 a 22 mm (Michener 2000). A grande maioria das espécies de Bombini são eussociais primitivas (Michener 2000; Silveira et al. 2002). Entretanto, algumas espécies, quase todas incluídas no subgênero *Psithyrus*, são parasitas sociais (Michener 2000; Silveira et al. 2002). Nestes casos, as fêmeas invadem os ninhos de outros Bombini e matam ou subjugam a rainha, de forma que as operárias hospedeiras permaneçam em atividade para manutenção da prole da espécie parasita (Michener 2000).

Os Bombini são um grupo de grande interesse econômico, tendo sido introduzido a uma grande variedade de culturas, tanto no campo quanto em casas de vegetação, para aumentar a produtividade pela polinização intensiva (Hanson & Gauld 1995).

Embora seja um grupo importante e bem estudado em alguns aspectos da biologia reprodutiva (ex. Michener 2000; Baer et al. 2003), nenhum trabalho foi realizado abrangendo a morfologia detalhada dos espermatozoides dos Bombini. Neste trabalho, descrevemos a estrutura e ultraestrutura dos espermatozoides de *Bombus (fervidobombus) atratus* por considerarmos que estudos desta natureza possam vir a contribuir para o maior conhecimento da biologia reprodutiva, taxonomia e filogenia do grupo.

MATERIAIS E MÉTODOS

Machos adultos de *Bombus (Fervidobombus) atratus* (Franklin) foram obtidos a partir de um ninho coletado na região de campo rupestre, no distrito de Lavras Novas, Ouro Preto (MG) e transferido para o Apiário Central da Universidade Federal de Viçosa, Viçosa (UFV), MG.

Microscopia de Luz

As vesículas seminais foram dissecadas e abertas sobre lâminas de vidro, para procedermos o espalhamento e fixação dos espermatozóides. Estes foram fixados com uma solução de glutaraldeído 2,5% em tampão cacodilato de sódio 0,1 M, pH 7,2. Após secagem em temperatura ambiente, as lâminas foram observadas em fotomicroscópio (Olympus, BX60), equipado com contraste de fase.

Para a medição nuclear, algumas destas lâminas foram coradas por 15 min. com DAPI (0.2 µg/ml 4,6-diamino-2-phenylindol) em tampão fosfato-salina. Após lavagem, as lâminas foram montadas com sacarose e examinadas em fotomicroscópio (Olympus, BX60), equipado com filtro de excitação BP360-370 nm.

Microscopia Eletrônica de Transmissão

Testículos e vesículas seminais foram dissecados e fixados por 3 horas em uma solução de glutaraldeído a 2,5% adicionado de 0,2% de ácido pícrico, 3% de sacarose e 5 mM de CaCl₂ em tampão de cacodilato de sódio 0,1 M, pH 7,2. O material foi pós-fixado em tetróxido de ósmio a 1% por 1-2 horas, desidratado em série crescente de acetona

e incluído em Epon. Os cortes foram contrastados com acetato de uranila 1% e citrato de chumbo 0,1%, e observados em Microscópio Eletrônico de Transmissão (Zeiss LEO 906).

Para detecção de proteínas básicas, foi utilizada a metodologia de E-PTA (ethanolic phosphotungstic acid). Neste caso, os testículos e vesículas seminais foram fixados apenas na solução de glutaraldeído, como descrito anteriormente. Após lavagem em tampão cacodilato e desidratação em etanol, o material foi tratado “em bloco” com PTA a 2% em etanol absoluto por 2 horas, na temperatura ambiente. Posteriormente procedemos a inclusão, corte e observação do material sem contrastação.

RESULTADOS

Os espermatozóides de *Bombus (Fervidobombus) atratus*, são células muito finas e alongadas, com, em média, 190 μm de comprimento (Fig. 2). Nos testículos, os espermatozóides estão organizados em feixes contendo 64 destas células (Fig. 3). Na vesícula seminal, os espermatozóides estão individualizados e dispostos aleatoriamente, embora alguns fragmentos dos feixes da organização testicular, possam eventualmente serem observados.

Os espermatozóides são constituídos pelas regiões de cabeça e flagelo (Fig. 2). A cabeça compreende o acrossomo e o núcleo, enquanto que o flagelo inclui o axonema, o adjunto do centríolo, os derivados mitocondriais e os corpos acessórios.

O acrossomo ocorre em bicamada, sendo constituído pela vesícula acrossomal e perforatorium (Figs. 4-5, 7). A vesícula acrossomal tem formato de cone e recobre o perforatorium até a extremidade anterior nuclear, onde o perforatorium é inserido (Fig. 4). Em corte transversal, tanto o perforatorium quanto a vesícula acrossomal são circulares na extremidade anterior (Figs. 5, 7). Entretanto, a vesícula acrossomal torna-se elipsoidal ao

longo da maior parte do comprimento, enquanto o perforatorium permanece circular (Figs. 5, 7). A vesícula acrossomal é fortemente E-PTA positiva e o perforatorium negativo (Fig. 7).

O núcleo é bastante alongado, homogêneo e compacto, medindo cerca de 15 µm de comprimento (Figs. 1, 3). A extremidade anterior nuclear é assimétrica (Fig. 4), sendo possível observar, em corte transversal desta região, simultaneamente, o perforatorium, parte do núcleo e parte da vesícula acrossomal (Fig. 7). O núcleo é observado negativo a levemente E-PTA positivo, e, dependendo do nível de secção transversal, este apresenta uma região central alongada intensamente marcada (Figs. 6-7). A extremidade posterior nuclear é afilada excentricamente, lateral ao encaixe da extremidade anterior do derivado mitocondrial maior e anterior à inserção do axonema (Figs. 5, 8).

O axonema apresenta o padrão de 9 + 9 + 2 microtúbulos, sendo 9 microtúbulos acessórios simples mais externos, 9 duplas e um par central (Fig. 10). Os microtúbulos acessórios e o material intertubular adjacente a eles, são E-PTA positivos, enquanto que as duplas são negativas e o par central aparece levemente contrastado (Figs. 15-17). Na região terminal do axonema, as duplas e o par central terminam simultaneamente, anteriores à finalização do acessórios (Figs. 20-22).

Os derivados mitocondriais são assimétricos em comprimento e diâmetro, com o derivado mitocondrial maior começando mais anteriormente, adjacente à extremidade posterior nuclear (Figs. 5, 8), e terminando, pouco depois do derivado mitocondrial menor (Figs. 18, 19). Em corte transversal, os derivados mitocondriais são elipsoidais, sendo o derivado maior levemente recurvado sobre o menor (Figs. 10, 14). A relação entre as áreas dos derivados mitocondriais, varia consideravelmente ao longo do flagelo. Dependendo do nível da secção transversal, a área do derivado maior pode exceder a área do menor em até

2,5 vezes (Figs. 10, 14, 17), enquanto que, na região de finalização, as áreas são praticamente equivalentes (Fig. 18). Ainda, em corte transversal, os derivados mitocondriais apresentam as seguintes regiões: (a) região circular, central e de menor eletrondensidade; (b) região dispersa adjacente ao axonema de maior eletrondensidade; (c) região de cristas mitocondriais, restrita à borda mais distal em relação ao axonema; (p) região de material paracristalino, exclusiva do derivado mitocondrial maior (Figs. 10, 16-17). As regiões a e p são fortemente E-PTA negativas (Figs. 16-17). Em corte longitudinal, as cristas mitocondriais são observadas perpendiculares ao eixo maior dos derivados e com espaçamento de 35 nm entre elas (Fig. 9).

O adjunto do centríolo é longo e está localizado entre o núcleo e o derivado mitocondrial menor (padrão assimétrico), paralelamente ao axonema e o derivado mitocondrial maior (Figs. 9, 11-13, 15-16). O adjunto do centríolo apresenta o formato aproximado de um cone, onde a extremidade anterior é mais afilada que a posterior. Assim, em corte transversal, ele é circular anteriormente (Figs. 11-12, 15), tornado-se aproximadamente triangular (Figs. 10, 13, 16). Independente do nível seccionado, o adjunto do centríolo tem área bastante inferior àquela do derivado mitocondrial maior (Figs. 10-13, 15-16). A extremidade posterior do adjunto do centríolo afila excentricamente junto à extremidade anterior do derivado mitocondrial menor (Fig. 9, setas). O adjunto do centríolo é E-PTA positivo, levemente marcado na região central (Figs. 15-16).

Os corpos acessórios, em corte transversal, têm formato aproximadamente triangular e estão localizados entre os derivados mitocondriais e o axonema, não estando presentes entre o axonema e adjunto do centríolo (Fig. 10). Os corpos acessórios marcam positivamente pela metodologia de E-PTA (Figs. 15-17).

DISCUSSÃO

O comprimento dos espermatozóides de *Bombus (Fervidobombus) atratus*, em média com 190 μm , está compreendido entre os valores descritos para *B. terrestris*, com 168 μm , *B. pascuorum*, com 181 μm e em *B. lapidarius*, com 230 μm (Quicke et al. 1992). Considerando as demais tribos corbiculadas, o comprimento dos espermatozóides em Bombini, é próximo ao registrado para Apini, cerca de 255 μm em *Apis mellifera* (ex. Cruz-Höfling et al. 1970) e Meliponini, onde estes variam de 80 a 300 μm , em *Nannotrigona punctata* e *Frieseomelitta varia*, respectivamente (Zama et al. 2003, no prelo). Entretanto é bastante inferior ao descrito para Euglossini, com cerca de 750 a 1500 μm , em *Eulaema nigrita* e *Euglossa mandibularis* (Zama et al. em preparação).

Os espermatozóides de *B. (Fervidobombus) atratus* apresentam muitas características ultraestruturais já observadas em outros Hymenoptera (Baccetti 1972; Wheeler et al. 1990; Quicke et al. 1992; Newman & Quicke 1998, 1999a, b, 2000; Jamieson et al. 1999; Lino-Neto et al. 1999, 2000a, b; Lino-Neto & Dolder 2001a, b, 2002; Zama et al. 2001, 2003, no prelo). Dentre elas, (1) acrossomo em bicamada, (2) núcleo bastante longo e compacto, (3) axonema com padrão 9 + 9 + 2 microtúbulos, (4) dois derivados mitocondriais, (5) dois corpos acessórios e (6) um longo adjunto do centríolo.

O acrossomo nas abelhas corbiculadas ocorre em bicamada, sendo constituído pela vesícula acrossomal recobrindo o perforatorium. Este padrão é comum nos Hymenoptera, tendo sido descrito em Symphyta (Quicke et al. 1992; Newman & Quicke 1999a), em Aculeata (Hoage & Kessel 1968; Cruz-Höfling et al. 1970; Lensky et al. 1979; Wheeler et al. 1990; Peng et al. 1992, 1993; Lino-Neto & Dolder 2002) e na vespa parasítica *Trissolcus basalis* (Scelionidae; Lino-Neto & Dolder 2001a). O acrossomo em tricamada apresenta uma bainha extra-celular recobrindo a vesícula acrossomal e a extremidade

anterior nuclear. Este padrão foi descrito em Parasítica (Quicke et al. 1992; Newman & Quicke 1998, 1999b; Lino-Neto et al. 1999, 2000a; Lino-Neto & Dolder 2001a). E, finalmente, *Telenomus podisi*, um Scelionidae, aparentemente não apresenta acrossomo (Lino-Neto & Dolder 2001a).

Embora todas as abelhas das tribos corbiculadas até agora estudadas apresentarem acrossomo em bicamada, uma diferença consistente pode ser observada em corte transversal, caracterizando pelo menos três padrões: (1) Em Bombini e Euglossini, o perforatorium é circular ao longo de todo seu comprimento, enquanto que a vesícula acrossomal é anteriormente circular, tornando-se elipsoidal em cerca de dois terços do comprimento (Zama et al. em preparação); (2) Em Meliponini, o perforatorium é circular ao longo de todo seu comprimento, enquanto que a vesícula acrossomal é anteriormente circular, tornando-se triangular (Zama et al. 2001, 2003, *no prelo*); (3) Em Apini, o perforatorium é retangular e a vesícula acrossomal é elipsoidal ao longo de todo seu comprimento (ex. Hoage & Kessel 1968; Cruz-Höfling et al. 1970; Lensky et al. 1979; Peng et al. 1992, 1993).

A morfologia da região nuclear é bastante semelhante nos corbiculados (Cruz-Höfling et al. 1970; Lensky et al. 1979; Peng et al. 1992, 1993; Zama et al. 2001). Entretanto, anteriormente, a região de transição entre acrossomo e núcleo é observada assimétrica e complementar nos Bombini, Euglossini (Zama et al. em preparação) e Apini (Cruz-Höfling et al. 1970; Lensky et al. 1979; Peng et al. 1992, 1993), de forma que em corte transversal, são observados simultaneamente núcleo, vesícula acrossomal e perforatorium (Figs. 4, 7). Já em Meliponini, estas extremidades são simétricas (Zama et al. 2001, 2003, *no prelo*).

O aspecto mais interessante da caracterização nuclear, foi observado no material tratado pela metodologia de E-PTA. Nos demais insetos conhecidos, o núcleo é homogêneo e E-PTA negativo (Báo 1991, 1996; Báo & Hamú 1993; Lino-Neto et al. 1999, 2000a, b; França & Báo 2000; Mancini 2002, informação pessoal). Entretanto, em Bombini (Fig. 6), Euglossini (Zama et al. em preparação) e Meliponini (Zama et al. 2003, no prelo), o núcleo é levemente positivo, e, dependendo do nível de secção transversal, este apresenta uma região central alongada intensamente marcada. Esta área poderia representar uma região de maior concentração de proteínas básicas, entretanto, nenhuma evidencia desta diferenciação pôde ser notada através de outras metodologias.

Nos Bombini, bem como nos demais corbiculados (Lino-Neto et al. 2000b, Zama et al. 2001, 2003, no prelo), o adjunto do centríolo é assimétrico. Este padrão também foi descrito nos Symphyta, exceto Siricoidea (Quicke et al. 1992; Newman & Quicke 1999a), nos Ichneumonoidea (Newman & Quicke 1998), nos Cynipoidea (Newman & Quicke 1999b) e nos Megalyroidea (Newman & Quicke 2000). Entretanto, no Symphyta *Tremex* sp (Siricoidea) (Newman & Quicke 1999a) e nas formigas (Wheeler et al. 1990; Lino-Neto & Dolder 2002), o adjunto do centríolo é anterior a ambos os derivados mitocondriais (simétrico). Já nos Chalcidoidea (Lino-Neto et al. 1999, 2000a; Lino-Neto & Dolder 2001b), esta estrutura envolve a região de transição “núcleo-flagelo”, projetando anteriormente, paralelo ao núcleo, e posteriormente, anterior a ambos os derivados mitocondriais. Finalmente, não foi observado adjunto do centríolo nos Scelionidae (Lino-Neto & Dolder 2001a).

O adjunto do centríolo varia nos corbiculados em pelo menos dois aspectos: (1) Morfologia da estrutura: em Bombini e Apini (Lino-Neto et al. 2000b), o adjunto do centríolo é afilado anteriormente, aumentado em área ao longo de seu comprimento, até

cerca de metade da área do derivado mitocondrial maior (ver Figs. 11, 12 e 13); em Meliponini esta estrutura é bastante regular, de forma que a área é aproximadamente a mesma ao longo de todo seu comprimento (Zama et al. 2001, 2003, no prelo); em Euglossini, o adjunto é bastante proeminente e, dependendo do nível de secção, supera a área do derivado mitocondrial maior. Nesta tribo, o adjunto do centríolo parece reduzir em área posteriormente, próxima a região de transição para derivado mitocondrial menor (Zama et al. em preparação). (2) Morfologia da região de transição entre adjunto de centríolo e derivado mitocondrial: em Bombini e Meliponini (Zama et al. 2001, 2003, no prelo), a extremidade posterior do adjunto do centríolo afila excentricamente junto à extremidade anterior do derivado mitocondrial menor (ver Fig. 9). Já em Apini e Euglossini, a extremidade posterior do adjunto do centríolo é aproximadamente côncava, recobrando a extremidade anterior do derivado mitocondrial menor (Lino-Neto et al. 2000b; Zama et al. em preparação).

Ainda, quanto à reação ao E-PTA, em Bombini e Apini, o adjunto do centríolo é positivo (Lino-Neto et al. 2000b), porém negativo em Meliponini (Zama et al. 2003, no prelo) e Euglossini (Zama et al. em preparação).

Os derivados mitocondriais são assimétricos em diâmetro em *Bombus* (*Fervidobombus*) *atratus*, assim como nos demais Aculeata, embora em Formicidae esta diferença seja bem menos acentuada (Rothschild 1955; Hoage & Kessel 1968; Cruz-Höfling et al. 1970; Lensky et al. 1979; Woyke 1984; Wheeler et al. 1990; Quicke et al. 1992, Peng et al. 1992, 1993; Lino-Neto et al. 2000b; Lino-Neto & Dolder 2002). Também são assimétricos os derivados dos Symphyta, exceto Siricoidea (Quicke et al. 1992; Newman & Quicke 1999a) e dos parasítica Megalyroidea (Newman & Quicke 2000), Cynipoidea (Quicke et al. 1992; Newman & Quicke 1999b) e Proctotrupeoidea (Quicke et

al. 1992). Já no Symphyta Siricoidea (Quicke et al. 1992; Newman & Quicke 1999a), nos Ichneumonoidea (Quicke et al. 1992) e nos Chalcidoidea (Lee & Wilkes 1965; Wilkes & Lee 1965, Hogge & King 1975; Lino-Neto et al. 1999, 2000a; Lino-Neto & Dolder 2001b), os derivados mitocondriais são equivalentes em diâmetro, sendo considerados portanto, simétricos. E, nos Scelionidae, apenas um derivado mitocondrial foi observado (Lino-Neto & Dolder 2001a).

Os derivados mitocondriais de todos os corbiculados até agora estudados são assimétricos em comprimento, de forma que o derivado mitocondrial maior começa antes e termina depois do que o derivado menor. Na região mais posterior do flagelo de Bombini, as áreas de secção transversa dos derivados mitocondriais são muito próximas, indicando que o derivado menor finaliza imediatamente antes que o maior. Já em Apini (Cruz-Höfling et al. 1970), Euglossini (Zama et al. em preparação) e Meliponini (Zama et al. 2001, 2003, no prelo), as áreas são ainda mais discrepantes do que em níveis superiores, indicando que o derivado menor finaliza bem antes que o maior. Ainda, os cortes transversais dos derivados mitocondriais de todos os corbiculados, apresentam as regiões **a**, **b**, **c** e **p**. Entretanto, estas apresentam reações diferentes frente à metodologia de E-PTA. Em Bombini, Euglossini (Zama et al. em preparação) e Apini (Lino-Neto et al. 2000b), a região **b** é fortemente positiva, enquanto que as regiões **a** e **p** são negativas. Já em Meliponini, todo o derivado mitocondrial é negativo, com uma marcação periférica na região **p** (Zama et al. em preparação).

O axonema deste Bombini apresenta o padrão 9+9+2 microtúbulos, como em todos os Hymenoptera conhecidos (Baccetti 1972; Jamieson et al. 1999). Na porção final do axonema deste Bombini, os microtúbulos da dupla e do par central são perdidos simultaneamente, antes da eliminação dos acessórios. Esta característica é compartilhada

com as demais abelhas corbiculadas (Zama et al. 2001, 2003, no prelo) e com as formigas (Lino-Neto & Dolder 2002) enquanto que nos parasítica são perdidos os acessórios, depois os centrais e finalmente as duplas (Lino-Neto et al. 1999, 2000a; Lino-Neto & Dolder 2001b). Quanto à marcação pela metodologia de E-PTA, nos corbiculados apenas os microtúbulos acessórios e o material intertubular são positivos (Lino-Neto et al. 2000b; Zama et al. em preparação), embora em Bombini, o par central também apresente uma marcação levemente positiva.

Os corpos acessórios são morfologicamente muito semelhantes entre os corbiculados conhecidos, bem como nos demais Hymenoptera (Rothschild 1955; Hoage & Kessel 1968; Cruz-Höfling et al. 1970; Lensky et al. 1979; Woyke 1984; Wheeler et al. 1990; Quicke et al. 1992; Peng et al. 1992, 1993; Jamieson et al. 1999, Newman & Quicke 1999a, b, 2000; Lino-Neto et al. 2000b, 2002; Lino-Neto & Dolder 2001a, 2002; Zama et al. 2001, 2003 no prelo). Entretanto, quando preparados pela metodologia de E-PTA, os corpos acessórios dos Bombini e Apini (Lino-Neto et al. 2000b) marcam positivamente enquanto que em Meliponini e Euglossini, esta estrutura é completamente E-PTA negativa (Zama et al. em preparação).

Concluindo, neste trabalho caracterizamos o espermatozóide de *Bombus (Fervidobombus) atratus* que, comparado com o descrito para os demais Hymenoptera, principalmente com as tribos mais próximas, Apini, Euglossini e Meliponini, demonstra um grande número de caracteres que poderão ser utilizados em futuros estudos filogenéticos.

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LEGENDA

Fig. 1. Núcleo marcado com DAPI. (1.206x)

Fig. 2 Espermatozóide observado em contraste de fase. (804x)

Figs. 3- 4 Corte longitudinal da região anterior do feixe testicular de espermatozoides. O parênteses na Fig. 3 marca a região ampliada na Fig. 4. As linhas tracejadas e numeradas da Fig. 4 equivalem aos níveis de secção transversal das Figs. 5 a 7. (*) material frouxo e elétron-denso que envolve o feixe. (3: 6.800x; 4: 15.000x)

Figs. 5- 7 Cortes transversais das regiões de acrosso e núcleo (Fig. 5, convencional e Figs. 6 e 7, E-PTA). Secções transversais: vesícula acrossomal (V) e perforatorium (P) em formato circular (1) e oval (2 e 3); (4) vesícula acrossomal, perforatorium e núcleo (N); (5) perforatorium na cavidade nuclear; (6) núcleo; (7) núcleo e derivado mitocondrial maior (D) (equivalente à linha tracejada da Fig. 8). Na Fig. 6, a seta indica a região central do núcleo mais intensamente E-PTA positiva. A vesícula acrossomal é E-PTA positiva, enquanto que o perforatorium é negativo. (5: 50.000x; 6: 40.000x; 7: 54.000x)

Fig. 8 Corte longitudinal da região de transição entre núcleo e flagelo. A seta indica a região do centríolo. (X) axonema. (28.000x)

Fig. 9 Corte longitudinal da região de transição entre adjunto do centríolo e derivado mitocondrial menor (d). A seta maior indica a intersecção das extremidades e a seta menor indica a região de cristas mitocondriais.

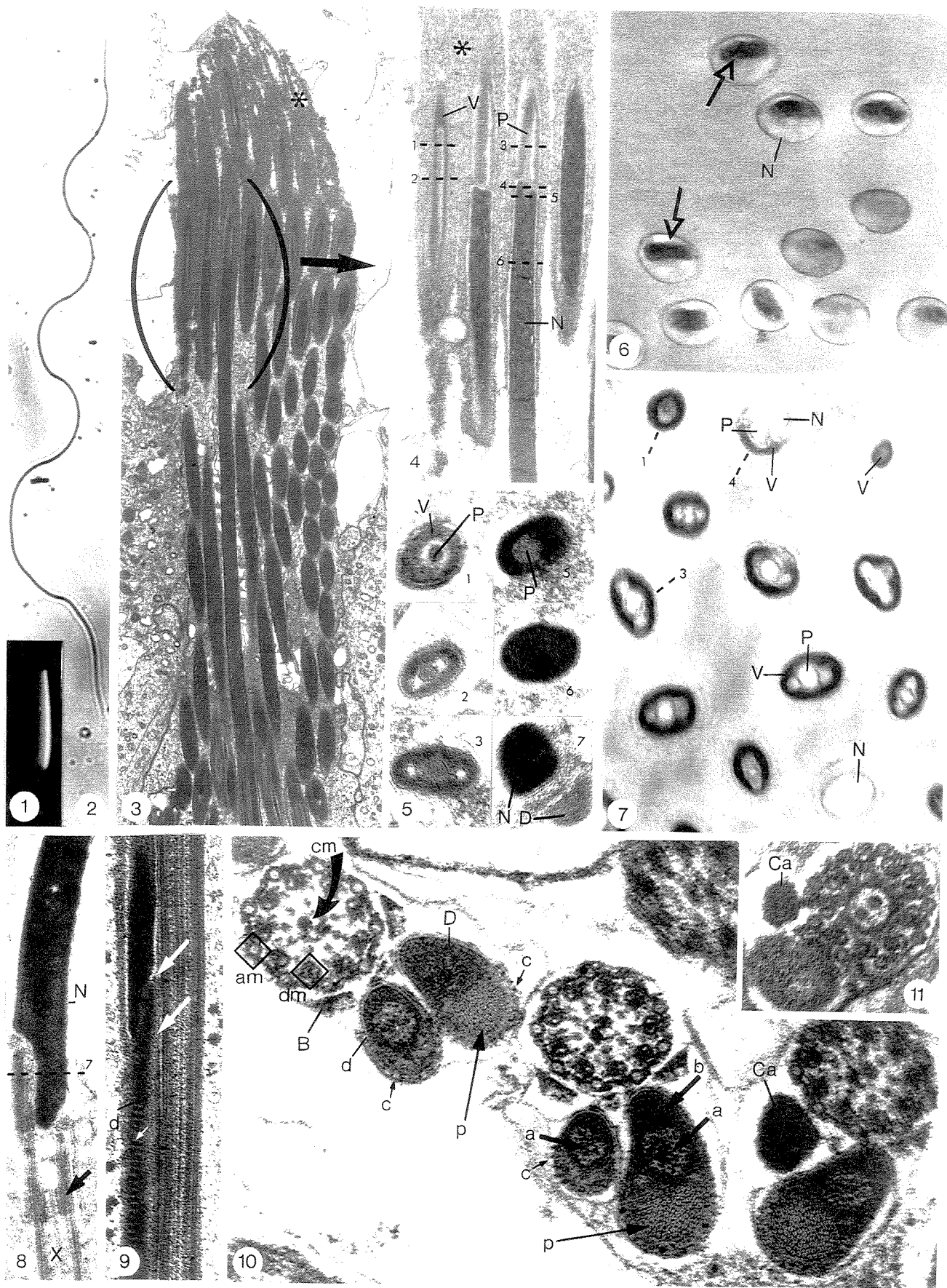
Fig. 10 Corte transversal dos flagelos na vesícula seminal. Observar no axonema os microtúbulos acessórios (am), das duplas (dm) e do par central (cm, na seta curva). Observar nos derivados as regiões a, b, de cristas (c) e de material paracristalino (p). (Ca)



adjunto do centríolo, (B) corpos acessórios, (d) derivado mitocondrial menor e (D) maior.

(100.000x)

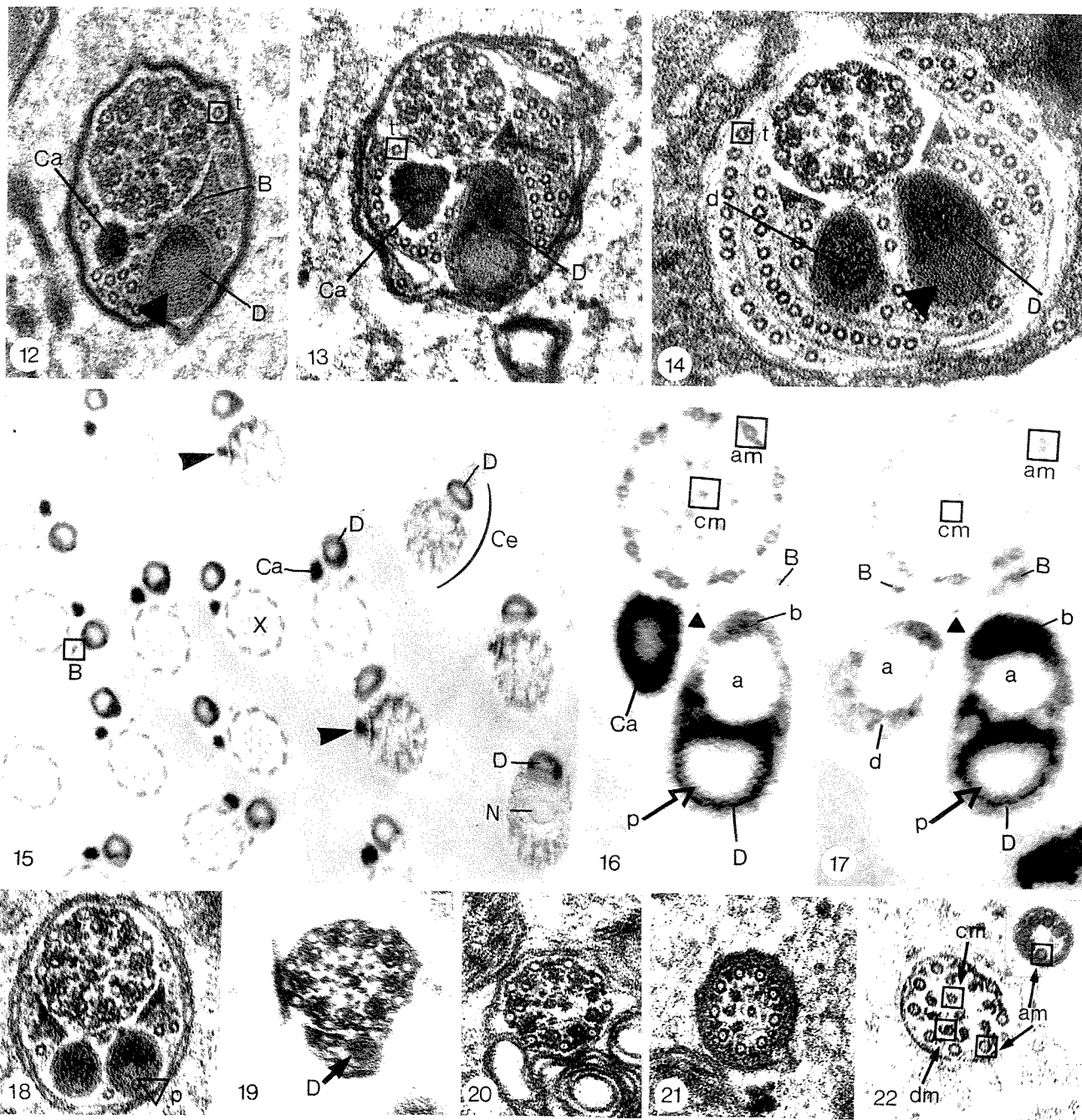
Fig. 11 Corte transversal da região mais anterior do flagelo, mostrando o início do adjunto do centríolo e derivado mitocondrial maior. (100.000x)



Figs. 12- 14 Cortes transversais do flagelo de espermatozóides nos cistos testiculares. Observar o aumento da área do adjunto do centríolo ao longo do comprimento do flagelo. (▶) indica o material paracristalino e nos detalhes, (t) indicam microtúbulos da manchete. (12: 83.000x; 13: 70.000x; 14: 85.000x)

Figs. 15- 17 Cortes transversais do flagelo marcado pela metodologia de E-PTA. Na Fig. 15, observar a origem do derivado mitocondrial maior (D) junto à projeção nuclear (N); a região de centríolo está indicada pelo parêntese; (cabeça de seta) origem do adjunto do centríolo ainda na altura do centríolo, com área bastante reduzida. Fig. 16, corte na região mais posterior do adjunto do centríolo e Fig. 17, na altura de ambos derivados mitocondriais. Observar que o adjunto do centríolo, microtúbulos acessórios e centrais, material intertubular, corpos acessórios e a região b dos derivados mitocondriais são fortemente E-PTA positivos, enquanto que os microtúbulos das duplas e as regiões a e p, são negativos. (15: 39.000x; 16, 17: 130.000x)

Figs. 18- 22 Cortes transversais da região posterior do flagelo. Na Fig. 18 os derivados mitocondriais maior e menor apresentam áreas aproximadamente equivalentes e na Fig. 19 observar que o derivado mitocondrial maior termina depois do menor. Nas figuras 20, 21 e 22, observar a sequência de finalização dos microtúbulos. (18, 19: 83.000x, 20, 21, 22: 63.000x)



1. DISCUSSÃO

Nesta etapa, pretendemos resumir as principais características dos espermatozóides em cada uma das tribos corbiculadas e compará-las com descrito para os demais Hymenoptera. Para tanto, utilizaremos as seguintes referencias bibliográficas:

SYMPHYTA:

- Symphyta - Quicke et al., 1992; Newman & Quicke, 1999a

APÓCRITA – ACULEATA:

- Apini (*Apis mellifera*) - Rothschild, 1955; Hoage & Kessel, 1968; Cruz-Höfling et al., 1970; Lensky et al., 1979; Woyke, 1984; Peng et al., 1992, 1993; Lino-Neto et al., 2000b.
- Meliponini - Zama et al., 2001, 2003 no prelo.
- Euglossini – manuscrito 2
- Bombini – Baer et al., 2003, manuscrito 3
- Formicidae - Thompson & Blum, 1967; Caetano, 1980; Wheeler et al., 1990; Lino-Neto & Dolder, 2002.

APÓCRITA – PARASITICA:

- Chalcidoidea - Lee & Wilkes, 1965; Wilkes & Lee, 1965; Hogge & King, 1975; Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b.
- Ichneumonoidea - Chauvin et al., 1988; Quicke et al., 1992.
- Cynipoidea - Quicke et al., 1992; Newman & Quicke, 1999b.
- Megalyroidea - Newman & Quicke, 2000.
- Scelionidae - Lino-Neto & Dolder, 2001a.
- Proctotrupeoidea – Quicke et al., 1992.

INFORMAÇÕES GERAIS: Jamieson, 1987, Quicke et al., 1992; Jamieson et al., 1999.

Estrutura

Os espermatozoides dos Hymenoptera são células finas e alongadas, apresentando comprimento total, geralmente entre 50 e 500 μm . Porém, algumas variações já foram descritas, de forma que os menores medem cerca de 8 a 12 μm e o maior, cerca de 620 μm . Nas abelhas corbiculadas, o comprimento desta célula está, em média, de acordo com o descrito para Hymenoptera: Em Meliponini estes variam de 80 a 300 μm , respectivamente em *Nannotrigona punctata* e *Frieseomelitta varia*. Em Bombini, os espermatozoides medem cerca de 168 μm em *Bombus terrestris*, 181 μm em *B. pascuorum*, 190 μm em *B. atratus* e 230 μm em *B. lapidarius*. Nos Apini, medem cerca de 255 μm em *Apis mellifera*. Entretanto, nos representantes da tribo Euglossini estudados neste trabalho, esta medida varia de 720 a 1500 μm , sendo considerados, portanto, os maiores espermatozoides conhecidos em Hymenoptera.

Nos Apidae corbiculados, os espermatozoides são tipicamente lineares, semelhante ao encontrado nos Symphyta, nos demais Aculeata e em alguns grupos de vespas parasíticas. Entretanto, os espermatozoides foram observados espiralados em Scelionidae e na maioria dos Chalcidoidea.

Acrossomo

O acrossomo nas abelhas corbiculadas ocorre em bicamada, ou seja, é composto pela vesícula acrossomal recobrando o perforatorium. A vesícula acrossomal ocorre anteriormente ao núcleo, não o sobrepondo. O perforatorium é observado encaixado em uma cavidade na extremidade anterior nuclear.

Embora o acrossomo nestas tribos guardem muitas semelhanças, quando estes são observados em diferentes níveis de cortes transversais, estes apresentam marcantes diferenças que caracterizam pelo menos três padrões: (1) Em Bombini e Euglossini, o perforatorium é observado circular ao longo de todo seu comprimento, enquanto que a vesícula acrossomal é anteriormente circular, tornando-se elipsoidal em cerca de dois terços do comprimento; (2) Em Meliponini, o perforatorium é circular ao longo de todo seu comprimento, enquanto que a vesícula acrossomal é anteriormente circular, tornando-se triangular; (3) Em Apini, o perforatorium é retangular e a vesícula acrossomal é elipsoidal ao longo de todo seu comprimento.

O acrossomo em bicamada já foi descrito em Symphyta, em Aculeata e na vespa parasítica *Trissolcus basalis* (Scelionidae). O acrossomo em tricamada apresenta uma bainha extra-celular recobrando a vesícula acrossomal e a extremidade anterior nuclear.

Este padrão foi descrito em Parasítica. E, finalmente, *Telenomus podisi*, um Scelionidae, aparentemente não apresenta acrossomo.

Núcleo

Esta estrutura é bastante conservada nos Hymenoptera. O núcleo é fino, alongado e, geralmente, bastante compacto. Entretanto, nos Meliponini *Melipona marginata* e *M. rufiventris* a cromatina aparece descompactada. Em *M. marginata* o aspecto é semelhante ao da cromatina em compactação das espermátides, já em *M. rufiventris* a cromatina está ainda mais frouxa, com regiões organizadas em grumos. Em *Solenopsis invicta* (Formicidae), foi observado no núcleo em corte longitudinal, diversas áreas de baixa densidade, porém não acreditamos que se trate da mesma característica.

A caracterização do núcleo das abelhas corbiculadas aqui estudadas frente a metodologia de E-PTA, demonstrando uma marcação não homogênea, ainda não foi observado em outros insetos. Tanto em Bombini quanto em Euglossini e Meliponini, o núcleo aparece levemente positivo, e, dependendo do nível de secção transversal, este apresenta uma região central alongada intensamente marcada. Esta área poderia representar uma região de maior concentração de proteínas básicas, entretanto, nenhuma evidência desta diferenciação pôde ser notada através de outras metodologias.

A morfologia nuclear é bastante semelhante nos corbiculados, entretanto duas diferenças podem ser observadas: (1) anteriormente, a região de transição entre acrossomo e núcleo é observada assimétrica e complementar nos Bombini, Euglossini e Apini, de forma que em corte transversal desta região, podem ser observados simultaneamente, vesícula acrossomal, perforatorium e núcleo. Já nos Meliponini, estas extremidades são simétricas; (2) posteriormente, a projeção nuclear termina antes do início do axonema nos Bombini, Euglossini e Meliponini, entretanto nos Apini, esta projeção está inserida no centro do arranjo centriolar.

Axonema

O axonema nos Hymenoptera apresenta, invariavelmente, o padrão de 9+9+2 microtúbulos. Este arranjo compreende, 9 microtúbulos acessórios, simples e mais externos; 9 duplas e um par central. Apesar da semelhança ultraestrutural do axonema nos Hymenoptera, pelo menos um aspecto têm sido variável nos grandes grupos Apocrita, não tendo sido descrito ainda para os Symphyta. Esta variação está na sequência de finalização dos microtúbulos do axonema na porção mais posterior do

flagelo. Nos Aculeata, os microtúbulos da dupla e do par central são perdidos simultaneamente, antes da eliminação dos acessórios. Enquanto que, nos Parasítica, são perdidos primeiro os acessórios, depois os centrais e finalmente as duplas.

Adjunto do Centríolo

Nos Hymenoptera, o adjunto do centríolo foi reconhecido recentemente, de forma que muitos trabalhos não tratam ou não identificam corretamente esta estrutura.

A morfologia do adjunto do centríolo varia na ordem de forma que pelo menos três padrões podem ser distinguidos: (1) Assimétrico: o adjunto do centríolo está localizado entre a extremidade posterior nuclear e a extremidade anterior de apenas um derivado mitocondrial (derivado menor). Este padrão é observado nas abelhas, nos Symphyta (exceto nos Siricoidea) e nos parasítica. (2) Simétrico: o adjunto do centríolo está interposto à extremidade posterior nuclear e a extremidade anterior de ambos os derivados mitocondriais. Este padrão é observado no Symphyta *Tremex* sp (Siricoidea) e nas formigas. (3) Projetado: o adjunto do centríolo se projeta anteriormente, paralelo ao núcleo, e posteriormente, anterior a ambos os derivados mitocondriais. Ainda, neste caso, o adjunto do centríolo preenche a região entre o núcleo e o flagelo. Este padrão foi observado apenas nos Chalcidoidea. E, finalmente, não foi observado adjunto do centríolo nos Scelionidae.

Nos corbiculados o adjunto do centríolo é assimétrico, entretanto algumas diferenças morfológicas podem ser observadas: (1) Morfologia: Nos Meliponini e nos Euglossini, o adjunto do centríolo é regular em dimensões (forma aproximada de bastão). Já nos Apini e Bombini, esta estrutura têm o diâmetro bastante reduzido na extremidade anterior, o qual aumenta ao longo do seu comprimento (forma de cone). (2) Conexão com o derivado mitocondrial: Nos Meliponini e Bombini a extremidade final do adjunto do centríolo é projetada lateralmente à extremidade anterior do derivado mitocondrial, enquanto que nos Apini e Euglossini, esta região é côncava e recobre a extremidade anterior do derivado mitocondrial menor.

Ainda, o adjunto do centríolo reage diferentemente à citoquímica de E-PTA nas tribos corbiculadas. Este é observado positivo nos Bombini e Apini, porém, é negativo em Meliponini e Euglossini.

Derivados Mitocondriais

Os derivados mitocondriais são consideravelmente assimétricos em diâmetro nos corbiculados. Nos Apini, Meliponini e Bombini, o derivado maior excede o menor em área de secção transversal em até duas vezes, ou pouco mais que isso. Já nos Euglossini, esta diferença é bem mais acentuada, onde o derivado maior a superar a área de secção transversa do menor, em mais de três vezes. Esta assimetria também é observada nos demais Aculeata, embora seja sutil nos Formicidae. Também são assimétricos os derivados dos Symphyta, exceto Siricoidea, e dos parasítica Megalyroidea, Cynipoidea e Proctotrupeoidea. Já no Symphyta Siricoidea, nos Ichneumonoidea e nos Chalcidoidea, os derivados mitocondriais são equivalentes em diâmetro, considerados, portanto, simétricos. Finalmente, os Scelionidae novamente constituem uma exceção, onde apenas um derivado mitocondrial foi observado.

Quando observados em corte transversal, os derivados mitocondriais das abelhas apresentam quatro regiões distintas: (a) região circular, central e de menor eletrondensidade. Nos Apini, esta região ocupa grande parte dos derivados, enquanto que nos Bombini, Euglossini e Meliponini, esta é mais reduzida; (b) região dispersa adjacente ao axonema de maior eletrondensidade; (c) região de cristas mitocondriais; Nos Apini, as cristas formam um semicírculo distribuídas em cerca de dois terços da periferia, enquanto que nos Bombini, Euglossini e Meliponini, estes aparecem restritos à periferia oposta ao axonema; (d) região de material paracristalino, exclusiva do derivado mitocondrial maior. Nos Euglossini, a região d é extremamente abundante quando comparada com as demais. A ocorrência do material paracristalino é comum nos Hymenoptera. Entre os grupos, o material paracristalino varia em abundância, na disposição no derivado mitocondrial em relação ao axonema e na ocorrência em ambos ou apenas um derivado mitocondrial. Por exemplo, nos Formicidae, estes estão presentes em ambos os derivados e estão localizados adjacentes ao axonema.

As quatro regiões dos derivados apresentam diferentes reações à metodologia de E-PTA. Em todas as tribos, a região de material paracristalino aparece negativa com marcação positiva por entre os cristais. Nos Apini, Bombini e Euglossini, a região a é positiva, enquanto b e c são negativas. Já nos Meliponini, todas as regiões são negativas, podendo ser observada apenas a periferia de d.

Os derivados mitocondriais de todos os corbiculados até agora estudados são assimétricos em comprimento, onde o derivado maior começa antes e termina depois que o menor. Na região mais posterior do flagelo, as áreas de secção transversal dos derivados são bastante discrepantes nos Apini, Euglossini e Meliponini, indicando que o derivado menor finaliza bem antes que o maior. Nos Bombini, estas áreas são muito próximas, indicando que o derivado menor finaliza imediatamente antes que o maior.

Corpos Acessórios

Os corpos acessórios também são estruturas de ocorrência, disposição e morfologia bastante conservada nos Hymenoptera. São estruturas alongadas e dispostas entre os derivados mitocondriais e axonema, ao longo de todo espermatozóide. Em corte transversal, são aproximadamente triangulares e variam nos grupos apenas quanto à proporção de sua área em relação a área das demais estruturas flagelares e, pelo menos nas abelhas, quanto à marcação pela metodologia de E-PTA. Nos Apini e Bombini, os adjunto do centríolo são positivos enquanto que nos Euglossini e Meliponini, esta estrutura é completamente E-PTA negativa.

2. CONCLUSÃO

Nossos resultados demonstraram que os espermatozoides dos Apidae corbiculados apresentam características, principalmente morfométricas, capazes de individualizar cada espécie ou gênero, como por exemplo, o comprimento total e nuclear desta célula. Outras características porém, são compartilhadas pelos representantes de cada uma das tribos, de forma que estas estabelecem um padrão típico para Meliponini, Euglossini, Bombini ou Apini.

Ainda, as tribos corbiculadas compartilham características que nos possibilitaram considerá-la como um grupo único, o qual pudemos analisar em relação aos demais Hymenoptera conhecidos. De forma que, acreditamos que a morfologia detalhada dos espermatozoides nos Hymenoptera, apresenta diversidade suficiente para fornecer caracteres válidos para estudos filogenéticos, pelo menos, nos táxons superiores.

Visto que as relações filogenéticas entre as quatro tribos de corbiculados ainda são um aspecto bastante discutido (Introdução - 4), neste trabalho nos propusemos testar a possibilidade de vir a utilizar as características estruturais e ultraestruturais dos espermatozóides desse grupo de abelhas que, associadas a outros caracteres, compusesse uma matriz capaz de fornecer, ou corroborar, uma proposta sobre as relações evolutivas nestes insetos.

Através dos resultados aqui obtidos foi possível estabelecer um padrão morfológico para os espermatozóides em cada uma das tribos. Inclusive, algumas diferenças específicas, principalmente morfométricas, puderam ser observadas. Como sugestão preliminar, listamos dezessete caracteres potenciais, considerando a ultraestrutura, a citoquímica de E-PTA e os comprimentos do núcleo e total deste tipo celular. Nós consideramos esta análise preliminar válida, pelo menos, para sugerir um agrupamento não polarizado dentro da tribo. Embora tenhamos obtido um cladograma referente a nossa matriz de caracteres, este resultado não é conclusivo, uma vez que a polarização foi feita em função de um ancestral hipotético como grupo externo. A caracterização desse ancestral hipotético foi feita com base em observações preliminares da morfologia dos espermatozóides de duas espécies da subfamília Apinae, *Centris* sp e *Tetrapedia* sp, associada a dados na literatura de outros Hymenoptera. Esta composição não é considerada ideal devido à grande distância filogenética de parte do grupo externo, a qual pode, em geral, apresentar características tão diversas que não podem ser comparadas. Finalmente, acreditamos que as características aqui consideradas são consistentes, mas que novos estudos ainda deverão ser feitos para incrementar esta listagem com novos caracteres.

As árvores (cladogramas) foram geradas utilizando algoritmos heurísticos de Hennig 86 (Farris, 1988), manejados com Tree Gardener 2.2.1 (Ramos, 1997). Os comandos utilizados nas análises foram *mhennig**, para obtenção de árvores mais curtas, e *bb**, baseado em trocas de ramos (branch swapping) para obtenção de árvores mais curtas. No caso da matriz 3, onde mais de um cladograma foi obtido, utilizamos o comando *nelson* para obtenção da árvore de consenso stricto.

2.1. DESCRIÇÃO DAS CARACTERÍSTICAS UTILIZADAS:

Descrição ilustrada no "ANEXO"

1. Morfologia da região anterior da vesícula acrossomal em corte transversal:
 - (0) circular;
 - (1) elipsoidal.
2. Morfologia da região posterior da vesícula acrossomal em corte transversal:
 - (0) elipsoidal;
 - (1) triangular.
3. Morfologia do perforatorium em corte transversal:
 - (0) circular;
 - (1) retangular.
4. Projeção longa e delgada da região anterior da vesícula acrossomal:
 - (0) ausente;
 - (1) presente.
5. Extremidade anterior nuclear:
 - (0) aproximadamente simétrico;
 - (1) assimétrico.
6. Projeção afilada da extremidade posterior nuclear:
 - (0) anterior ao arranjo centriolar do axonema;
 - (1) encaixada no centro do arranjo centriolar do axonema.
7. Morfologia do adjunto do centríolo:
 - (0) regular ao longo do comprimento;
 - (1) afilada na região anterior.
8. Região de conexão do adjunto do centríolo ao derivado mitocondrial menor:
 - (0) extremidade posterior do adjunto do centríolo côncava recobrimdo a extremidade anterior do derivado mitocondrial menor;
 - (1) extremidade posterior do adjunto do centríolo afilada e projetada lateralmente à extremidade anterior do derivado mitocondrial menor.
9. Morfologia do derivado mitocondrial maior:
 - (0) elipsoidal (de ovalado a alongado);
 - (1) piriforme.

10. Abundância de material paracristalino:

- (0) pouco abundante;
- (1) extremamente abundante.

11. Razão entre derivados mitocondriais:

- (0) derivado mitocondrial maior com área até 2,5 vezes maior que a área do derivado menor;
- (1) derivado mitocondrial maior com área superior a 2,5 vezes a área do derivado menor;

12. Relação entre as áreas dos derivados mitocondriais na região de posterior do flagelo:

- (0) derivado mitocondrial maior com área extremamente maior que a área do menor, até a finalização deste.
- (1) derivado mitocondrial maior com área próxima a área do menor, até a finalização quase que simultânea de ambos.

13. Disposição das cristas mitocondriais nos derivados:

- (0) Forma de semi-círculo ocupando cerca de dois terços da periferia dos derivados;
- (1) Restrita à periferia oposta ao axonema.

14. Reação dos corpos acessórios ao tratamento com E-PTA:

- (0) negativa;
- (1) positiva.

15. Reação da região b dos derivados mitocondriais ao tratamento com E-PTA:

- (0) positiva;
- (1) negativa.

16. Comprimento nuclear dos espermatozóides:

- (0) até 20 μm
- (1) superior a 20 μm

17. Comprimento total dos espermatozóides:

- (0) até 500 μm ;
- (1) superior a 500 μm .

2.2. MATRIZ DE CARACTERES:

MATRIZ 1: Considerando todos os 17 caracteres propostos.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
GE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E ₁	0	0	0	0	1	0	0	0	1	1	1	0	1	0	0	1	1
E ₂	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	1	1
B	0	0	0	0	1	0	1	1	0	0	0	1	1	1	0	0	0
M	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0
A	1	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0

Onde, GE: grupo externo; E: Euglossini (E₁: *Euglossa* e E₂: *Eufriesea*, *Exaerete*, *Eulaema*); B: Bombini; M: Meliponini; A: Apini (*Apis mellifera*)

MATRIZ 2: Desconsiderando a duplicidade do caracter 10 em Euglossini:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
GE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	1	0	0	0	1	1	0	1	0	0	1	1
B	0	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0
M	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0
A	1	0	1	1	1	1	1	0	0	0	0	0	1	0	0	0

Onde, GE: grupo externo; E: Euglossini; B: Bombini; M: Meliponini; A: Apini (*Apis mellifera*)

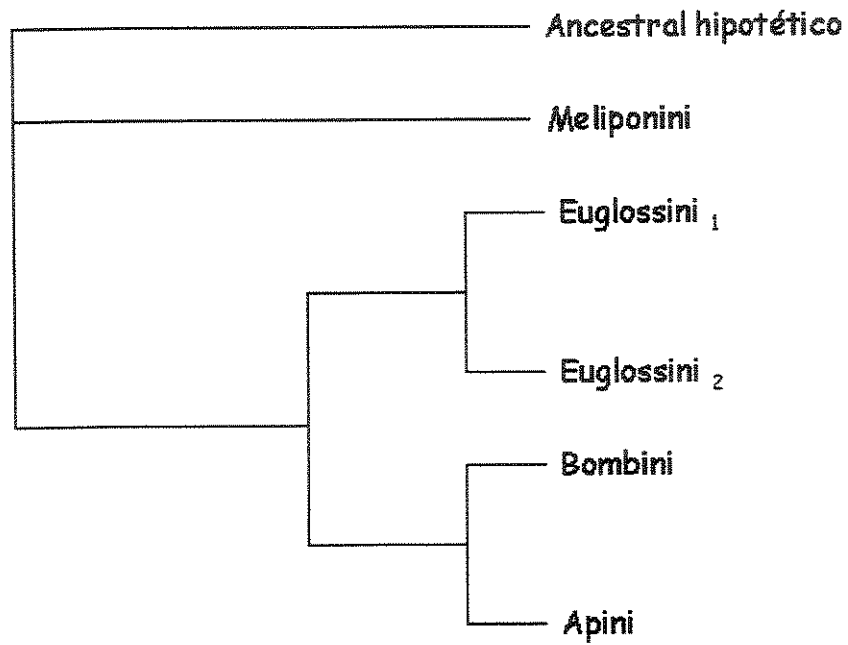
MATRIZ 3: Desconsiderando os caracteres de citoquímica de E-PTA (14 e 15):

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
GE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E ₁	0	0	0	0	1	0	0	0	1	1	1	0	1	1	1
E ₂	0	0	0	0	1	0	0	0	0	1	1	0	1	1	1
B	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0
M	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
A	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0

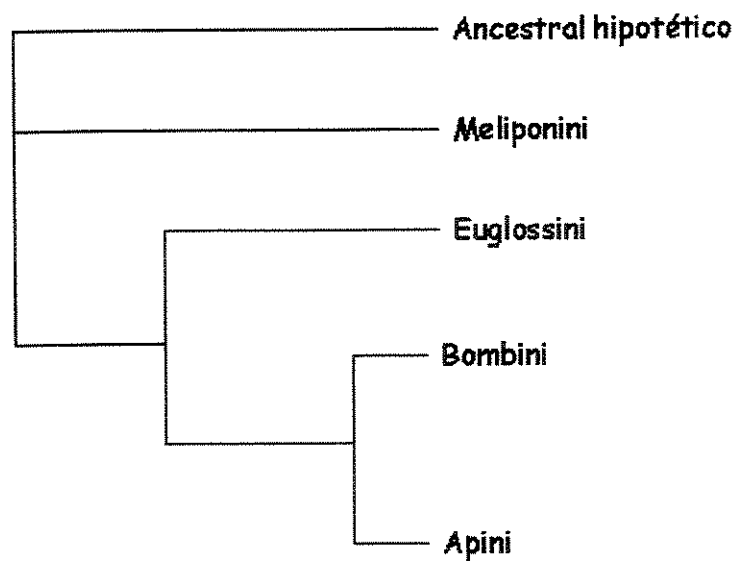
Onde, GE: grupo externo; E: Euglossini (E₁: *Euglossa* e E₂: *Eufriesea*, *Exaerete*, *Eulaema*); B: Bombini; M: Meliponini; A: Apini (*Apis mellifera*)

2.3. CLADOGRAMAS

CLADOGRAMA 1: referente à matriz 1

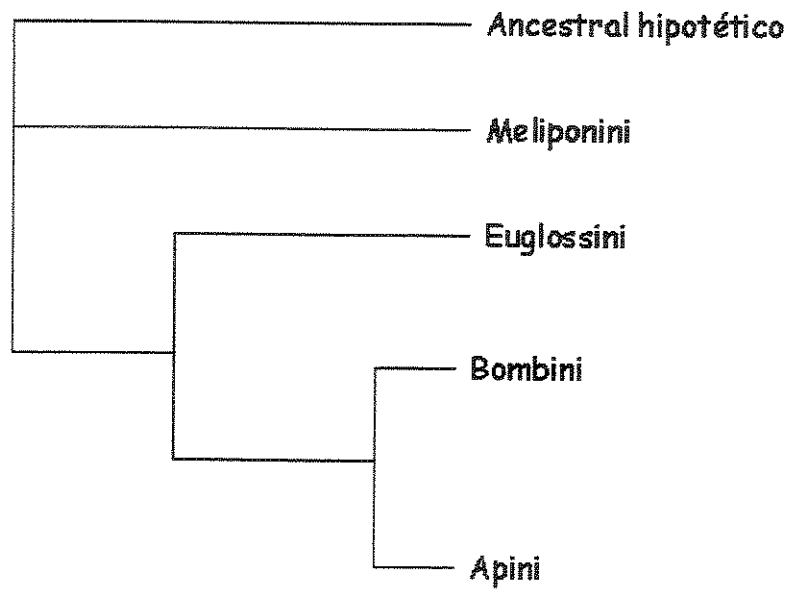


Ou, resumidamente:



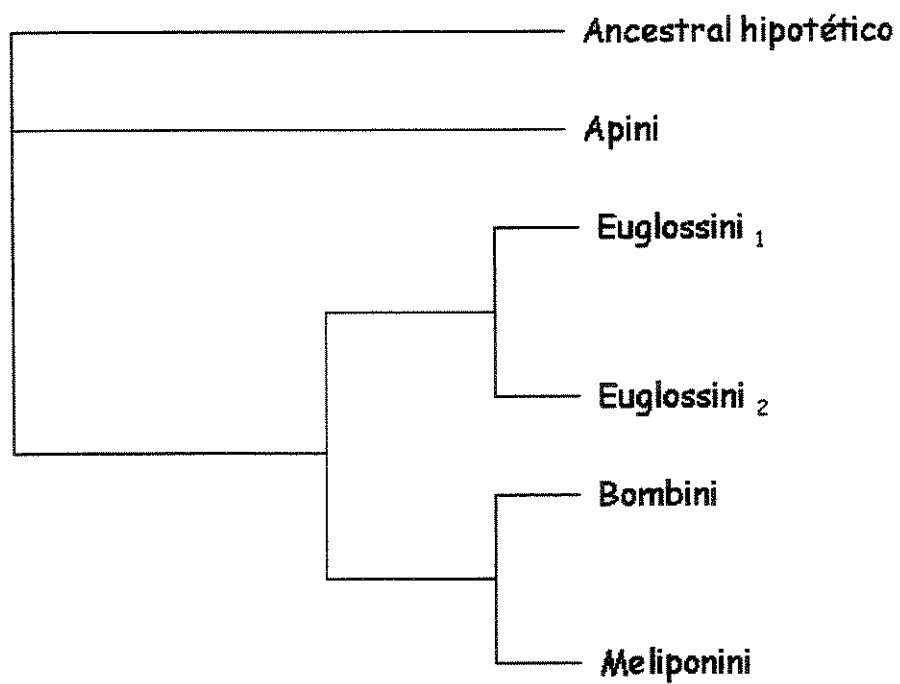
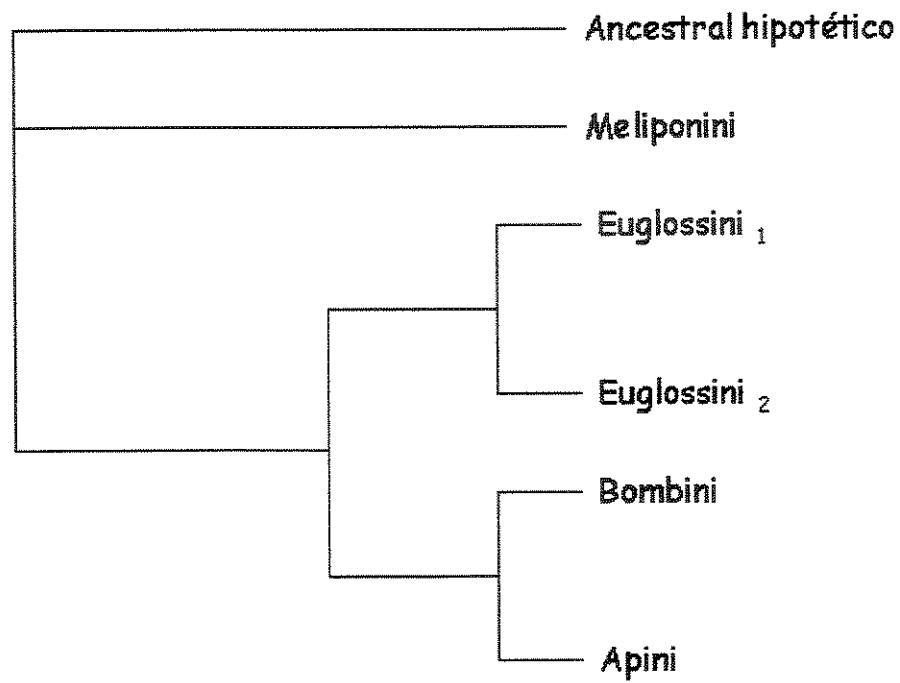
(Meliponini (Euglossini (Bombini + Apini)))
consistência: 19, Ci: 89, Ri: 77

CLADOGRAMA 2: referente à matriz 2

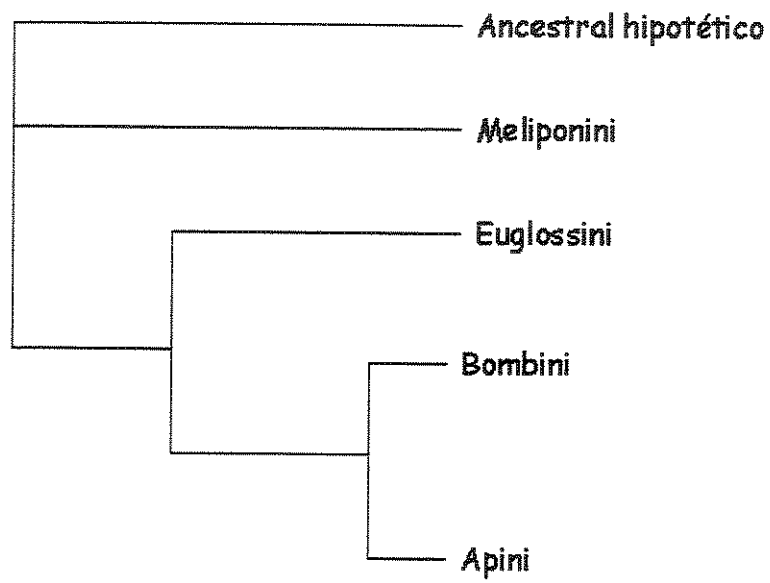


(Meliponini (Euglossini (Bombini + Apini)))
consistência: 18, Ci: 88, Ri: 60

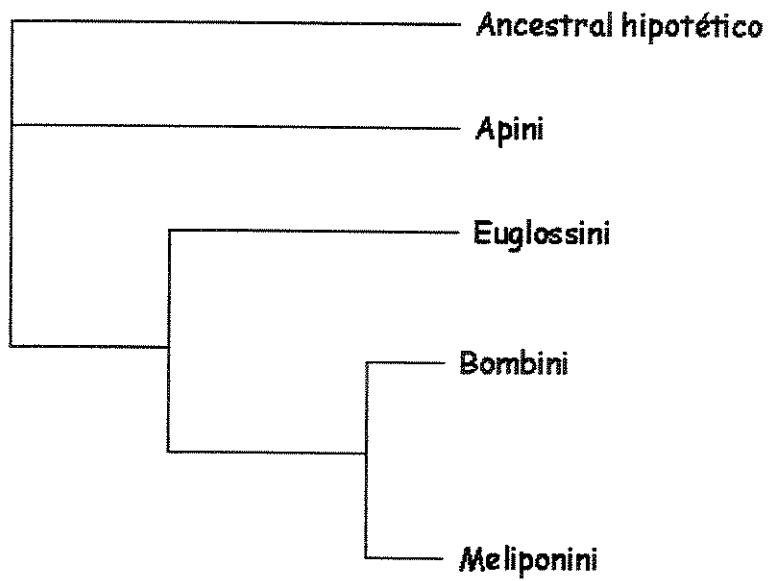
CLADOGRAMA 3: referente à matriz 3



Ou , resumidamente:

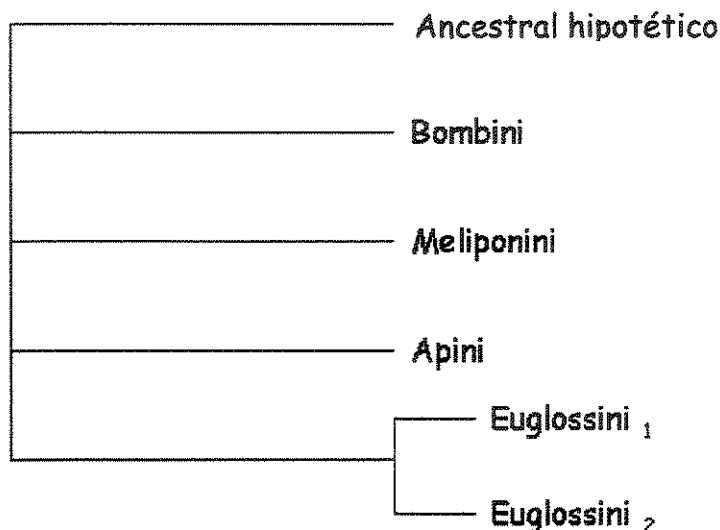


(Meliponini (Euglossini (Bombini + Apini)))
consistência: 17, Ci: 88, Ri: 75



(Apini (Euglossini (Bombini + Meliponini)))
consistência: 17, Ci: 88, Ri: 75

A matriz 3 forneceu dois cladogramas, de forma que a seguinte árvore de consenso stricto foi obtida:



2.4. CONCLUSÃO

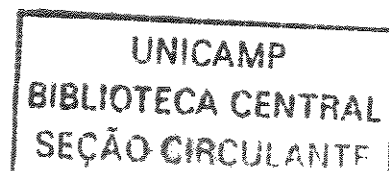
Nossas análises indicam que Meliponini seria o grupo mais basal dos corbiculados, seguido por Euglossini, que por sua vez, seria irmão do clado formado por Bombini e Apini. Esquematicamente, nossos dados sugerem (Meliponini (Euglossini (Bombini + Apini))). Esta disposição não concorda com nenhuma das diversas hipóteses propostas atualmente (Michener, 1944, 1990; Winston & Michener, 1977; Kimsey, 1984; Plant & Paulus, 1987; Sheppard & McPheron, 1991; Cameron, 1991, 1993; Prentice, 1991; Roig-Alsina & Michener, 1993; Chavarria & Carpenter, 1994; Mardulyn & Cameron, 1999; Koulianos et al., 1999; Cameron & Mardulyn, 2001; Silveira et al., 2002; Noll, 2002). Acreditamos que nossos dados deverão ser associados aos dados de morfologia somática, comportamento e biologia molecular, para que uma nova matriz possa ser proposta e que resulte em uma hipótese mais consistente para o grupo. Assim, consideramos que os dados da morfologia dos espermatozoides são potencialmente úteis para futuras análises filogenéticas.

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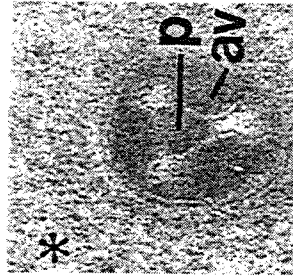
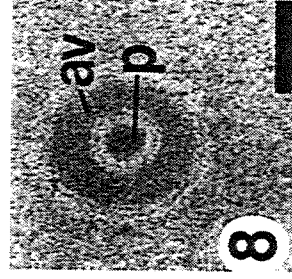
As imagens apresentadas neste anexo pertencem aos seguintes trabalhos:

- Apini – Peng et al., 1992, 1993;
Lino-Neto et al., 2000b;
- Meliponini – Zama et al., 2001, 2003 no prelo;
- Euglossini – manuscrito 2;
- Bombini – manuscrito 3.

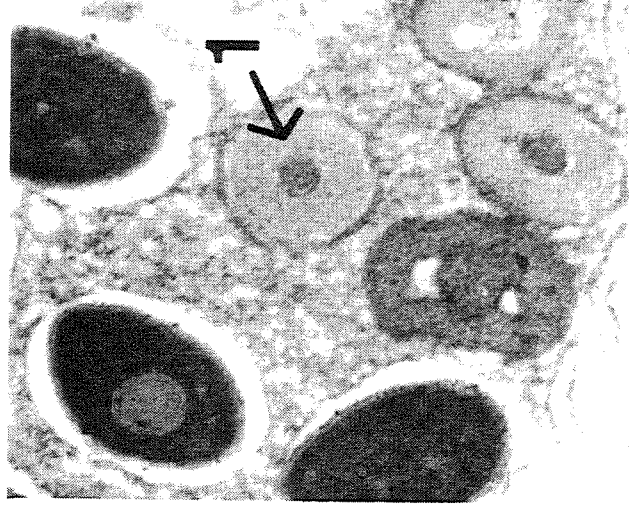
1. Morfologia da região anterior da vesícula acrossomal em corte transversal

(0) circular

(1) elipsoidal



Meliponini

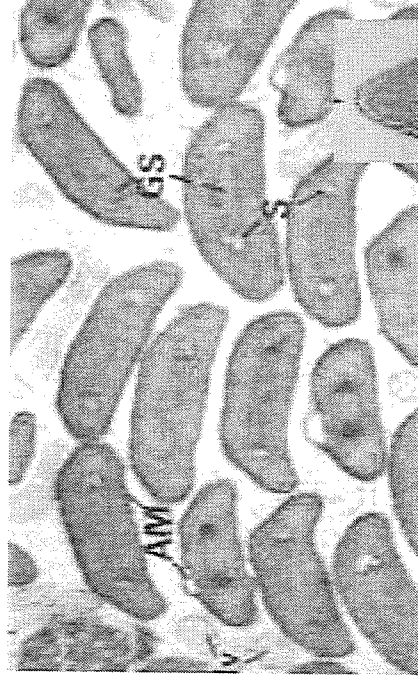


Euglossini + Bombini

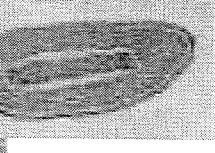
2. Morfologia da região posterior da vesícula acrossomal em corte transversal

(0) elipsoidal

(1) triangular



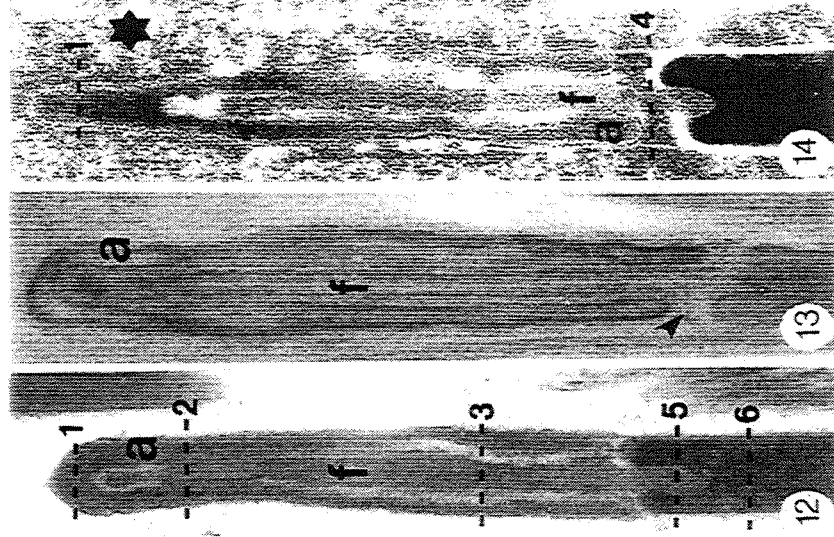
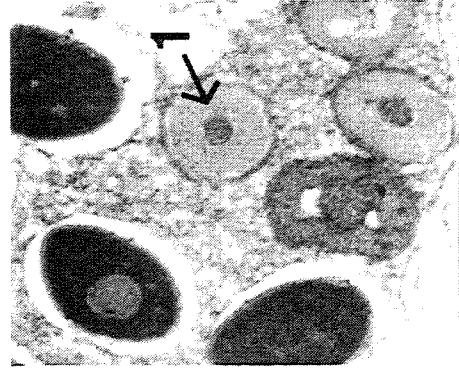
Apini



3. Morfologia do perforatorium

(0) circular

(1) elipsoidal

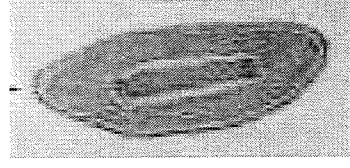
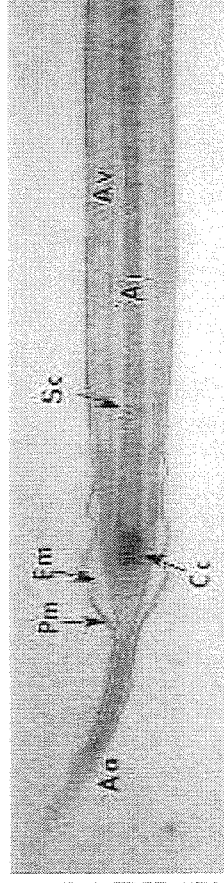


Meliponini +
Euglossini +
Bombini

4. Projeção longa e delgada da região anterior da vesícula acrossomal

(0) ausente

(1) presente

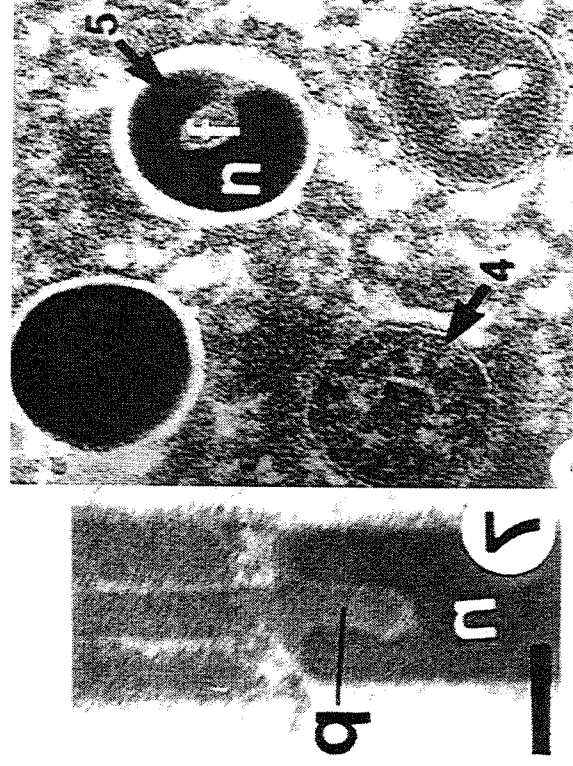


Apini

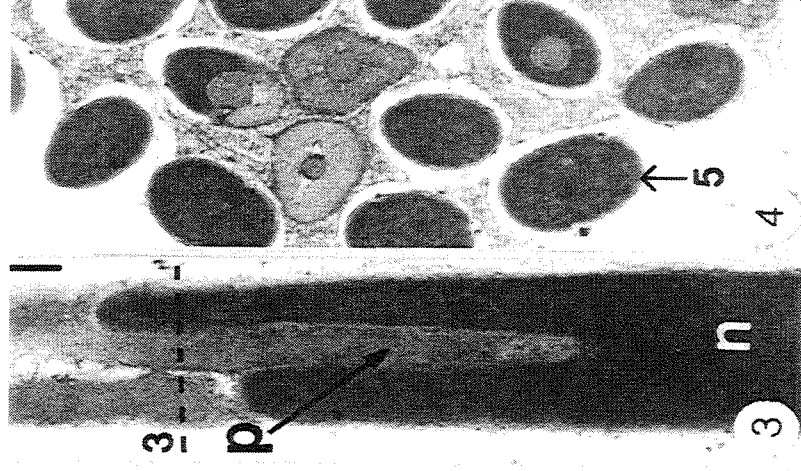
5. Extremidade anterior nuclear

(0) aproximadamente simétrico

(1) assimétrico



Meliponini + Apini



Euglossini + Bombini

6. Projeção afilada da extremidade posterior nuclear

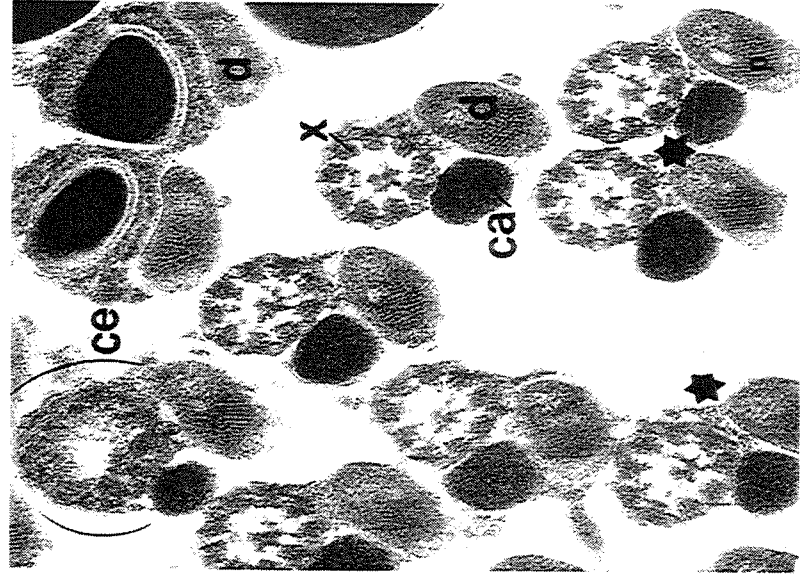
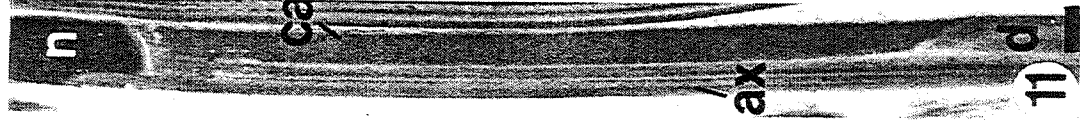
(0) anterior ao centríolo

(1) encaixada no centro do centríolo

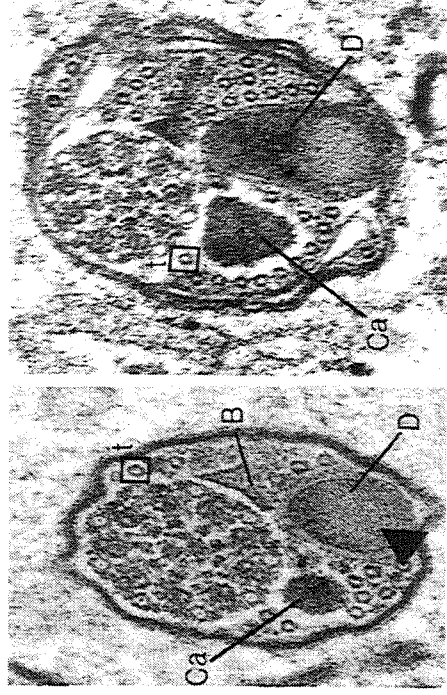


7. Morfologia do adjunto do centríolo

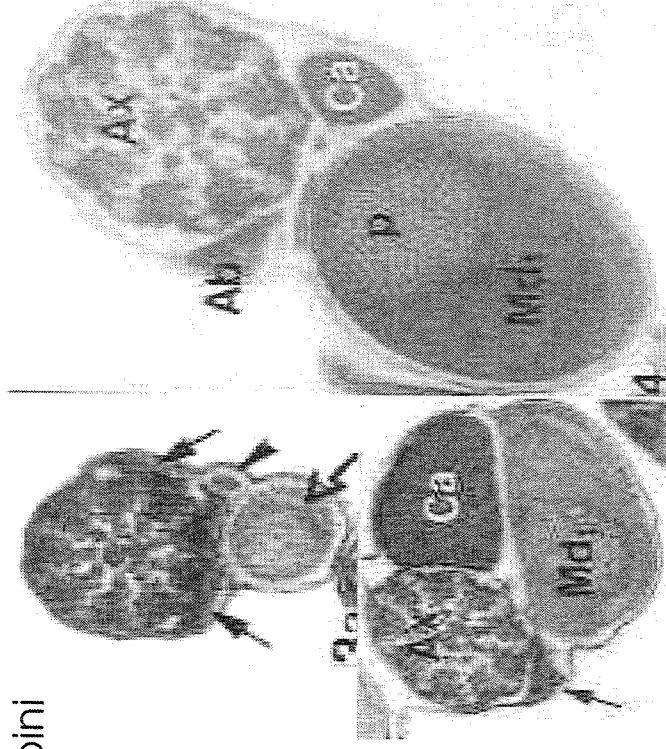
- (0) regular ao longo do comprimento
- (1) afilada na região anterior



Meliponini + Euglossini



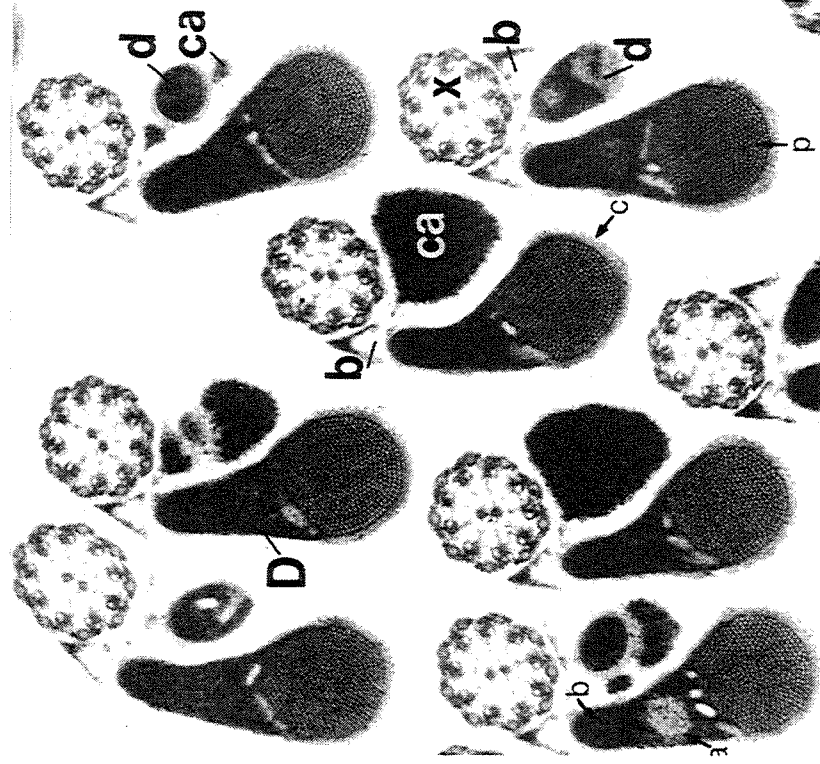
Bombini
+ Apini



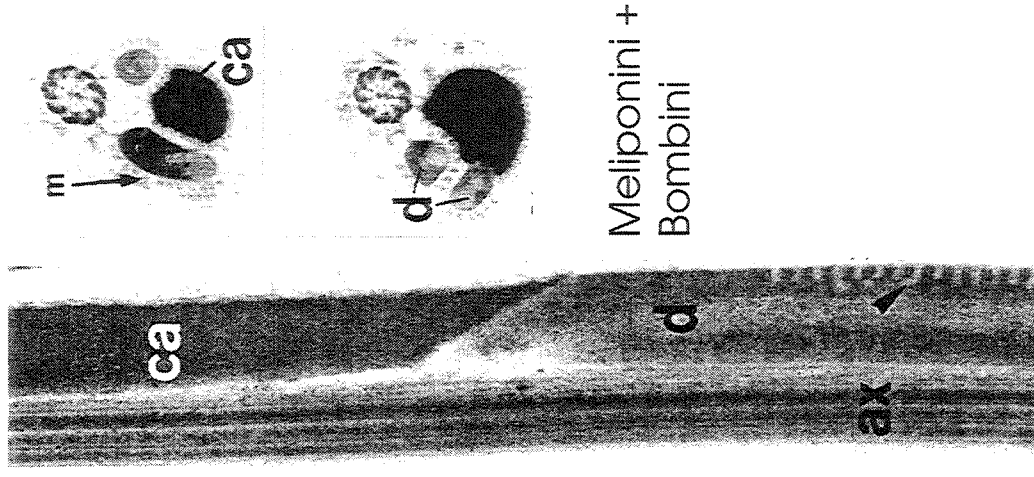
8. Conexão do adjunto do centríolo e derivado menor

(0) côncava

(1) projetada lateralmente



Apini +
Euglossini



Meliponini +
Bombini

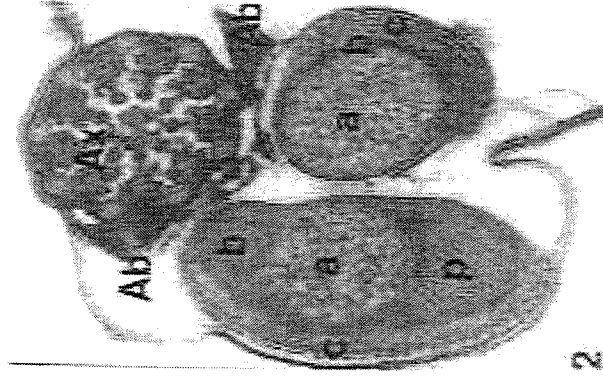
9. Morfologia do derivado mitocondrial maior 10. Abundância de material paracristalino

(0) elipsoidal (ovalado ou alongado)

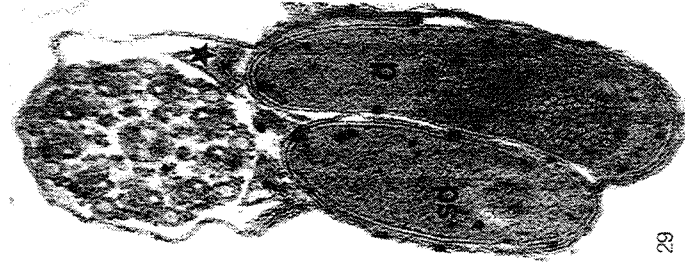
(0) pouco abundante

(1) periforme

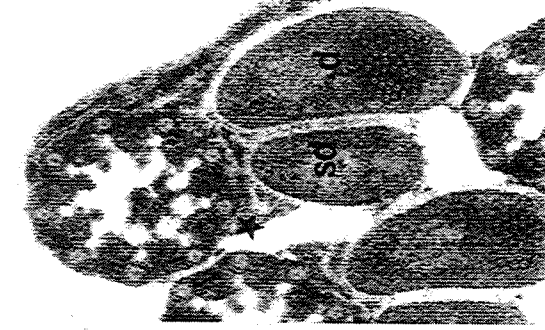
(1) extremamente abundante



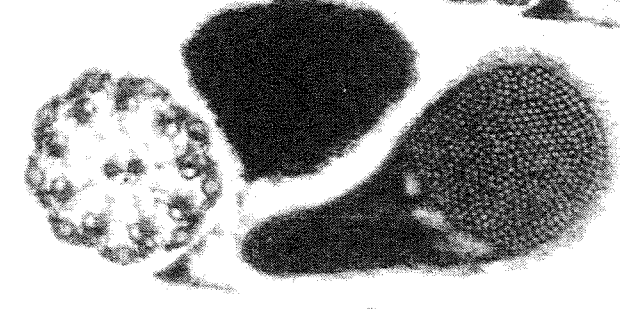
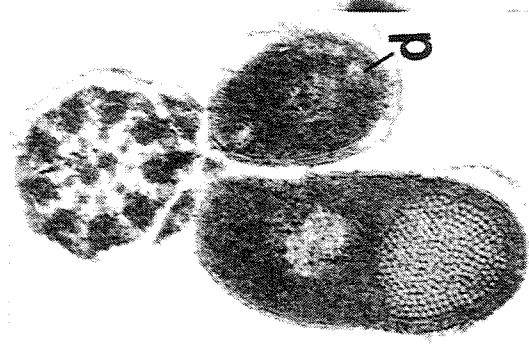
Apini



Meliponini +
Bombini



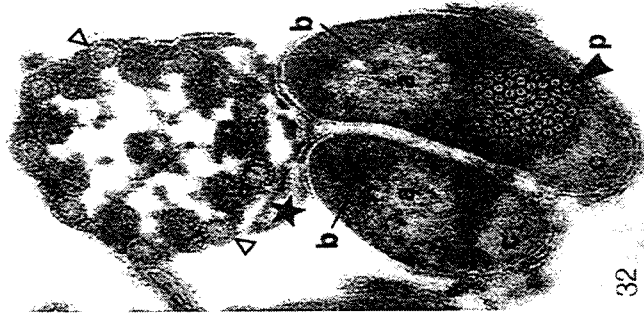
Euglossini



11. Razão entre derivado mitocondrial

(0) maior até 2,5x maior que o menor

(1) maior superior à 2,5x



Apini +
Bombini +
Meliponini

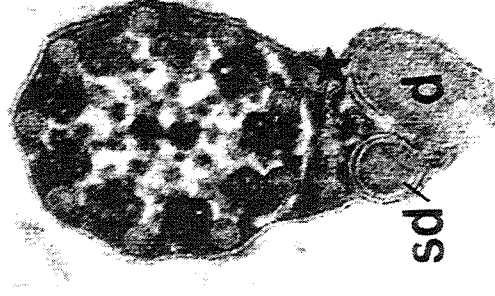


Euglossini

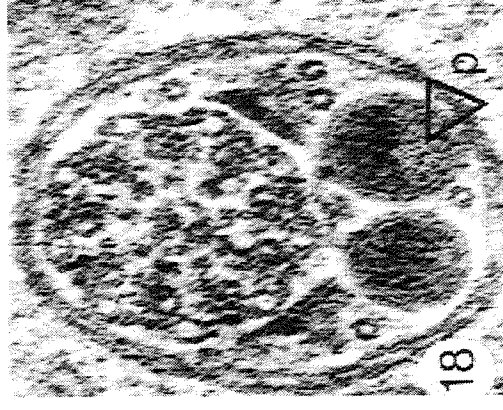
12. Relação entre as áreas dos derivado na região posterior do flagelo

(0) derivado maior o supera menor

(1) derivados quase equivalentes



Apini +
Euglossini +
Meliponini

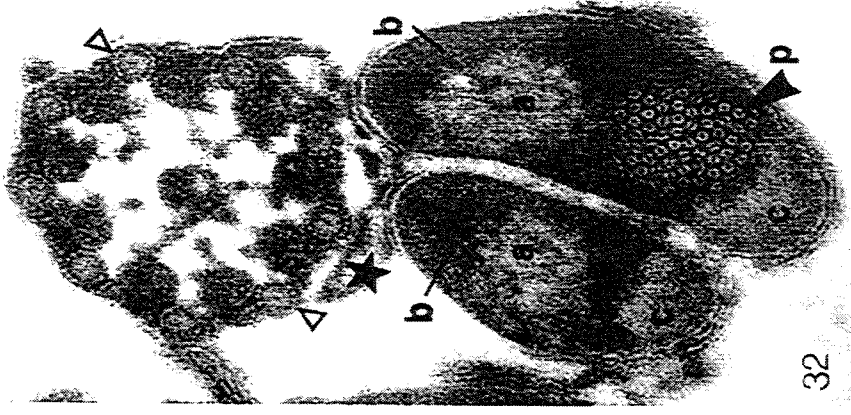


Bombini

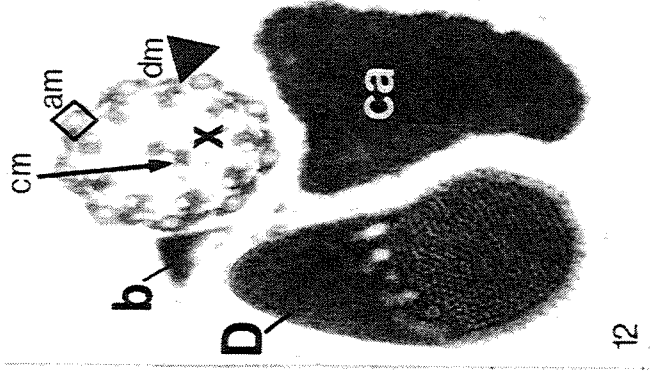
13. Disposição das cristas mitocondriais

(0) forma de semi-círculo ocupando 2/3 da periferia dos derivados

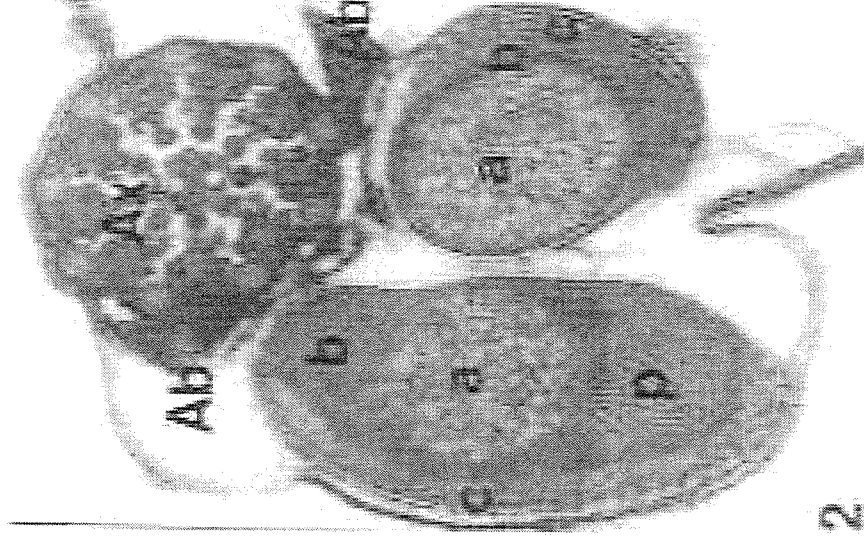
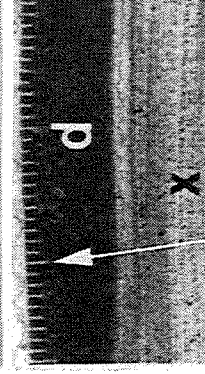
(1) restrita à periferia oposta ao axonema



32



12

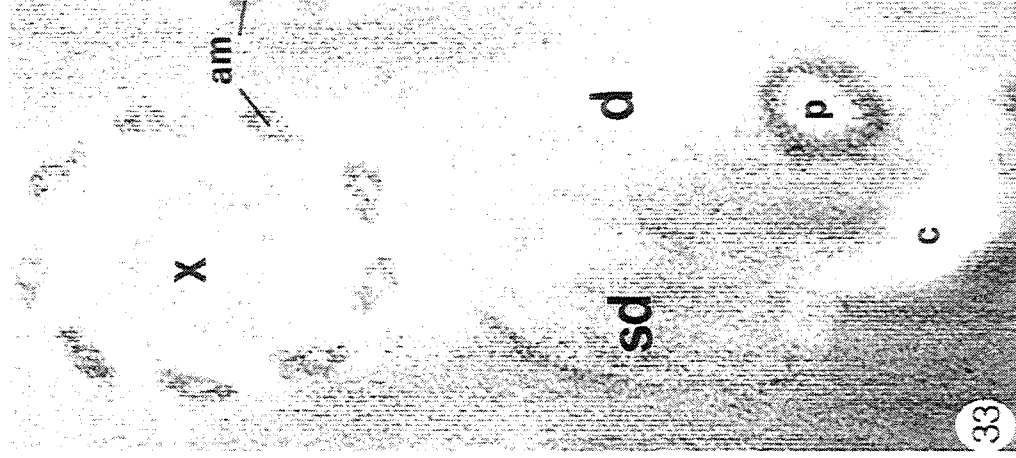


2

Bombini + Euglossini+ Meliponini

Apini

14. Corpos acessórios X E-PTA



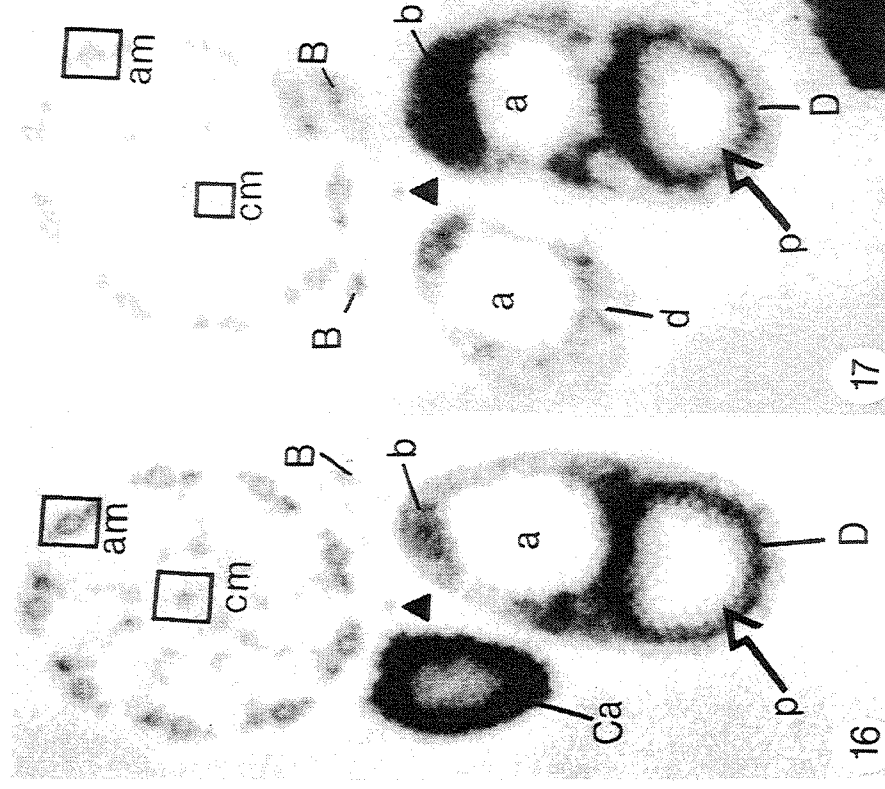
(0) negativo

(1) positivo

Meliponini

15. Região **b** X E-PTA

(0) positivo (1) negativo



Apini + Bombini + Euglossini

16. Comprimento nuclear

(0) até 20 μm

(1) maior que 20 μm

17. Comprimento total

(0) até 500 μm

(1) maior que 500 μm

Euglossini

