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Protocols

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Amyl acetate: an alternative technique to dry mount Chalcidoidea (Hymenoptera) from alcohol, faster and inexpensively

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Ethanol (EtOH) 70% is commonly used in collections to preserve and store many unprepared soft-bodied Chalcidoidea. Specimens air-dried directly from alcohol, however, often suffer from collapse of some body parts, making subsequent observation of their morphology difficult or even impossible. We propose an inexpensive method for processing and dry-mounting specimens of Chalcidoidea, using a chemical process including amyl acetate. Four treatments using amyl acetate at different concentrations and exposure times were evaluated for specimens of Eulophidae, Mymaridae, Encyrtidae, Aphelinidae, Pteromalidae, and Trichogrammatidae. Treatment with amyl acetate resulted in specimens of consistently higher quality. Based on our results, treatment of specimens for 1 h with 50:50 amyl acetate and ethanol mixture, followed by treatment for 1 h with 100% amyl acetate, yielded specimens adequate for morphological observations for most of the families. Further experiments are required, however, to optimize this approach for Trichogrammatidae and Eulophidae. This method is a relatively simple, inexpensive, and safe alternative to other methods commonly used for restoring Chalcidoidea preserved in alcohol.

Key words: insect preservation, collection, soft body, point-mounting

Resumen

El etanol (EtOH) al 70% se usa comúnmente en colecciones para preservar y almacenar muchos Chalcidoidea de cuerpo blando no preparados. Sin embargo, los especímenes secados al aire directamente del alcohol a menudo sufren el colapso de algunas partes del cuerpo, lo que dificulta o incluso imposibilita la observación posterior de su morfología. Nosotros proponemos un método económico para procesar y montar en seco especímenes de Chalcidoidea, utilizando un proceso químico que incluye acetato de amilo. Se evaluaron cuatro tratamientos con acetato de amilo a diferentes concentraciones y tiempos de exposición para Eulophidae, Mymaridae, Encyrtidae, Aphelinidae, Pteromalidae y Trichogrammatidae. El tratamiento con acetato de amilo dio como resultado especímenes de calidad superior. Según nuestros resultados, el tratamiento de las muestras durante 1 hora con una mezcla de acetato de amilo y etanol al 50:50, seguido de un tratamiento de 1 hora con acetato de amilo al 100 %, produjo muestras adecuadas para las observaciones morfológicas de la mayoría de las familias. Sin embargo, se requieren más experimentos para optimizar este enfoque para Trichogrammatidae y Eulophidae. Este método es una alternativa relativamente simple, económica y segura a otros métodos comúnmente utilizados para restaurar Chalcidoidea conservados en alcohol.

Palabras clave: Preservación de insectos, colección, cuerpo-suave, montaje en triángulo.

Introduction

Most Hymenoptera are safely preserved in 70% ethanol prior to dry-mounting specimens for morphological observation (van Achterberg 2009). However, many soft-bodied insects preserved in EtOH suffer a partial to complete collapse of body parts when air-dried from ethanol (Heraty and Hawks 1998), making it difficult for taxonomic studies. Chalcidoidea are especially susceptible to implosion, given their relatively thin and/or lightly sclerotized cuticle (Quicke et al. 1999, Noyes 2003).

Different methodologies have been utilized to dry Chalcidoidea. Critical point drying (CPD) using liquid CO_2 (Gordh and Hall 1979) is widely used to retrieve a large number of soft-bodied specimens from ethanol and prevent the collapse of specimens, including muscles and nerves (Heraty and Hawks 1998, Quicke et al. 1999). However, this is an expensive method to employ routinely given the initial cost of equipment (±$10.000 US) and the necessity to obtain CO_2 tanks (Heraty and Hawks 1998, Aguiar 2012).

Alternatives to CPD have been proposed, including freeze-drying (Flaschka and Floyd 1969, Nagy 2010), electric air pump (Aguiar 2012), and chemical desiccation. For the latter, chemical substances such as xylene (van Achterberg 2009), ethyl acetate (Vockeroth 1966), hexane (Quicke et al. 1999), methyl cellosolve (Bowles 2021), and hexamethyldisilazane (HMDS) (Heraty and Hawks 1998) are solvents that replace the alcohol in the specimen's tissues. Then the solvent volatilizes, leaving samples in a dry state (Bowles 2021). However, many of these chemical substances have toxic hazardous compounds (Sabrosky 1966, Vockeroth 1966, Truman 1968, Gordh and Hall 1979, Brown 1993, Aguiar 2012, Ali et al. 2021).

In Chalcidoidea, HMDS is one of the most accepted chemicals used for drying tiny wasps (Brown 1993), with similar results to CPD, preventing specimens collapse, maintaining body parts for visual examination, and significantly reducing costs (Heraty and Hawks 1998). Although HMDS is less expensive than CPD, its price is still prohibitively high for small laboratories in areas far from distribution centers, and its use requires extreme caution (National Center for Biotechnology Information 2023).

The AXA method (Alcohol/Xylene-Amyl acetate) is also commonly used for drying Hymenoptera with comparable results to HMDS. Preparation time, however, can take 2 to 5 days, but large quantities of specimens can be treated at once (van Achterberg 2009). Sample is filled with a mixture of 40% xylene and 60% absolute alcohol; after 1–3 days, this mixture is replaced by amyl acetate, then for 1 to 2 days, the specimens can be prepared (van Achterberg 2009). Notwithstanding a less expensive and less time-consuming method, biodiversity research needs to further optimize sample preparation due to large numbers of specimens that are collected in the field, and which need to be dry-mounted (Aguiar 2012).

Some of these drying methods, such as ethyl acetate, formaldehyde, and ethylene glycol, used to prepare insect samples for morphological identification, often prevent DNA analysis and extraction from those samples (Dillon et al 1996). We propose here an alternative and more economical method to prepare soft-bodied insects for dry-mounting. This method allows for alcohol-preserved specimens, to be dried for mounting, with minimal collapse and to be conserved properly for taxonomic research based on molecular sequences and morphological characters.

Experimental Design

All specimens were initially killed and preserved in 96% EtOH. Three hundred specimens representing the families Eulophidae, Mymaridae, Encyrtidae, Aphelinidae, Pteromalidae, and Trichogramatidae with soft-bodies, were chosen for evaluating the Amyl acetate technique in four treatments (Table 1).

Ten specimens per family (60 individuals by treatment) were immersed in each amyl acetate treatment solution (Table 1). After the allotted time had passed, each sample was removed from each solution with a brush and extended in card paper opaline for drying. Once ten minutes elapsed, specimens were point-mounted for morphological examination (Fig. 1), which was performed by an observer who remained unaware of the treatment, so as to avoid researcher bias. Finally, all material examined was deposited in the Laboratorio de Entomología Universidad de la Amazonia – LEUA (http://rnc.humboldt.org.co/admin/index.php/registros/detail/1893).

Specimens were scored as collapsed and not collapsed, based only on the softer body parts. A score of 'not collapsed' means that the specimen is sufficiently well-preserved to allow for taxonomic identification, that is, collapsed of not more than one body part or slight distortion, acceptable for museum collections. A score of collapse would be typical of air-dried specimens taken from alcohol, that is, challenging to identify, with large and discernible morphological changes, particularly of antenna and metasoma, both of which have remarkable features for identifying micro wasps.

A multiple correspondence analysis (MCA) was performed using the statistical package 'ade4' (Dray and Dufour 2007) to explore relationships between families studied and collapse specimen status for each treatment. Mosaic graphs were made using the 'vcd' package (Friendly and Meyer 2015), in which Pearson's residuals are used to shade and visualize independence test results, showing cells that contribute chi-square test significance, this information was used to determine associations between collapses specimen status and treatment regardless of specimen´s family. All analyses were performed in R language software, version 4.2.1 (R Core Team 2020).

Protocol

1. With a pin or brush takes out the specimens from the ethanol (96%) (Fig. 1).
2. In a glass container (avoid plastic vials which are solvable in amyl acetate) put the sample in a mixture of 50% amyl acetate and 50% ethanol 96% for at least 1 or 2 hours. Less fragile taxa (i.e., Pteromalidae and Encyrtidae) may skip this step and pass directly to step 3.
3. Transfer the specimen to amyl acetate (100%). Kept it by at least 1 or 2 h. However, time can change according to the taxa, and it is kept safe even for one night into the solution.
4. Removed the specimen from the solution of amyl acetate.

Table 1. Evaluated treatments for soft-bodied Chalcidoidea dried using amyl acetate at different concentrations and exposure times

Treatment	*Description*
T0	control absolute (alcohol preserved)
T1	50% alcohol + 50% amyl acetate (1 h) then 100% amyl acetate (1 h)
T2	50% alcohol + 50% amyl acetate (2 h) then 100% amyl acetate (2 h)
T3	100% amyl acetate (1 h)
T4	100% amyl acetate (2 h)

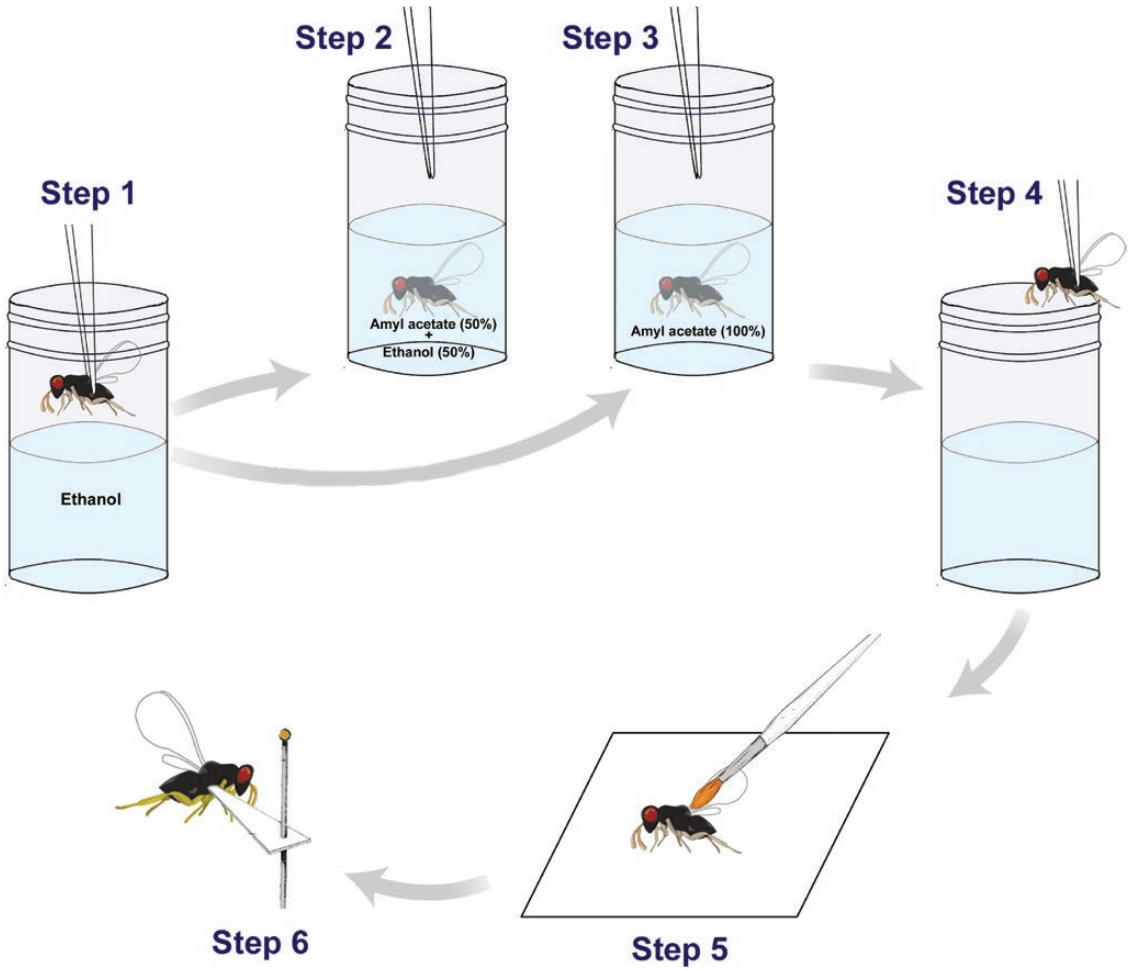

Fig. 1. Protocol scheme for dry-mounting using amyl acetate solution.

5. In an opaline paper card extend the specimen with a brush. If the wings are not well extended, add a drop of amyl with the tip of a brush and extended it. (Opaline is recommended because it quickly absorbs fluids of the specimen and allows proper handling of the sample).
6. Once the specimen is dry, the specimen is pointed or card-mounting for examination. Pinning should be done not later than 20 min after taking out the amyl acetate to avoid losing parts of the body and be able to reposition appendages during pinning.

Results

Three hundred specimens were qualitatively assessed to determine specimens degree of collapse. We found that very soft-bodied specimens under amyl drying technique generally performed well through treatments, compared to air-drying straight from alcohol, which usually tends to be disastrous as almost all body parts of insects under this treatment collapse (Fig. 2). In general, for all treatments families such as Aphelinidae (Fig. 2B), Encyrtidae (Fig. 2D), Trichogrammatidae (Fig. 2F), Eulophidae (Fig. 2H), and Mymaridae (Fig. 2J) presented a better appearance once mounted on cardstock points because several of its structures remained safe once air-dried. An evaluation of collapsed vs. non-collapsed Pteromalidae revealed few differences. Pteromalid specimens tend to be more sclerotized and do not collapse as easily as families mentioned above (Fig. 2K, L).

For the statistical analysis, we evaluate proportion of collapsed samples among treatments evaluated independently of family (Fig. 3). Multiple correspondence analysis (MCA) between Chalcidoidea families (Hymenoptera) and their reaction to treatments (Fig. 4), and proportion between collapsed and not collapsed specimens by family (Fig. 5) were performed. In first analysis, it is observed that cell's width is proportional to number of cases registered by category in each evaluated treatment. For T0, proportion of collapsed individuals compared to others is significantly higher (Fig. 3). Accordingly, treatment that guarantees adequate specimens for anatomical and morphological studies of Chalcidoidea specimens is T1, followed by T3, T2, and T4 (Fig. 3).

Consistent with above, the MCA plots explained 37.4% of data variability in its first two dimensions. Collapsed specimens of Trichogrammatidae and Eulophidae families were associated with T2. On the other hand, low contribution of variables T1_no and T3_no to MCA dimensions formation is because most of Chalcidoidea (Hymenoptera) specimens did not present collapses in two variables, which allows us to infer that T1 would be adequate to avoid collapse followed by T3 (Fig. 4).

Proportion of specimens 'collapsed' vs. 'not collapsed', plotted by taxonomic families shows that Encyrtidae specimens presented the best reaction when placed in amyl acetate given that 100% of all samples placed in T1, T2, and T3 maintained anatomical structures duly formed for their conservation (Fig. 5). Moreover, Pteromalidae, Mymaridae, Encyrtidae, and Aphelinidae families did not show any collapse with T2.

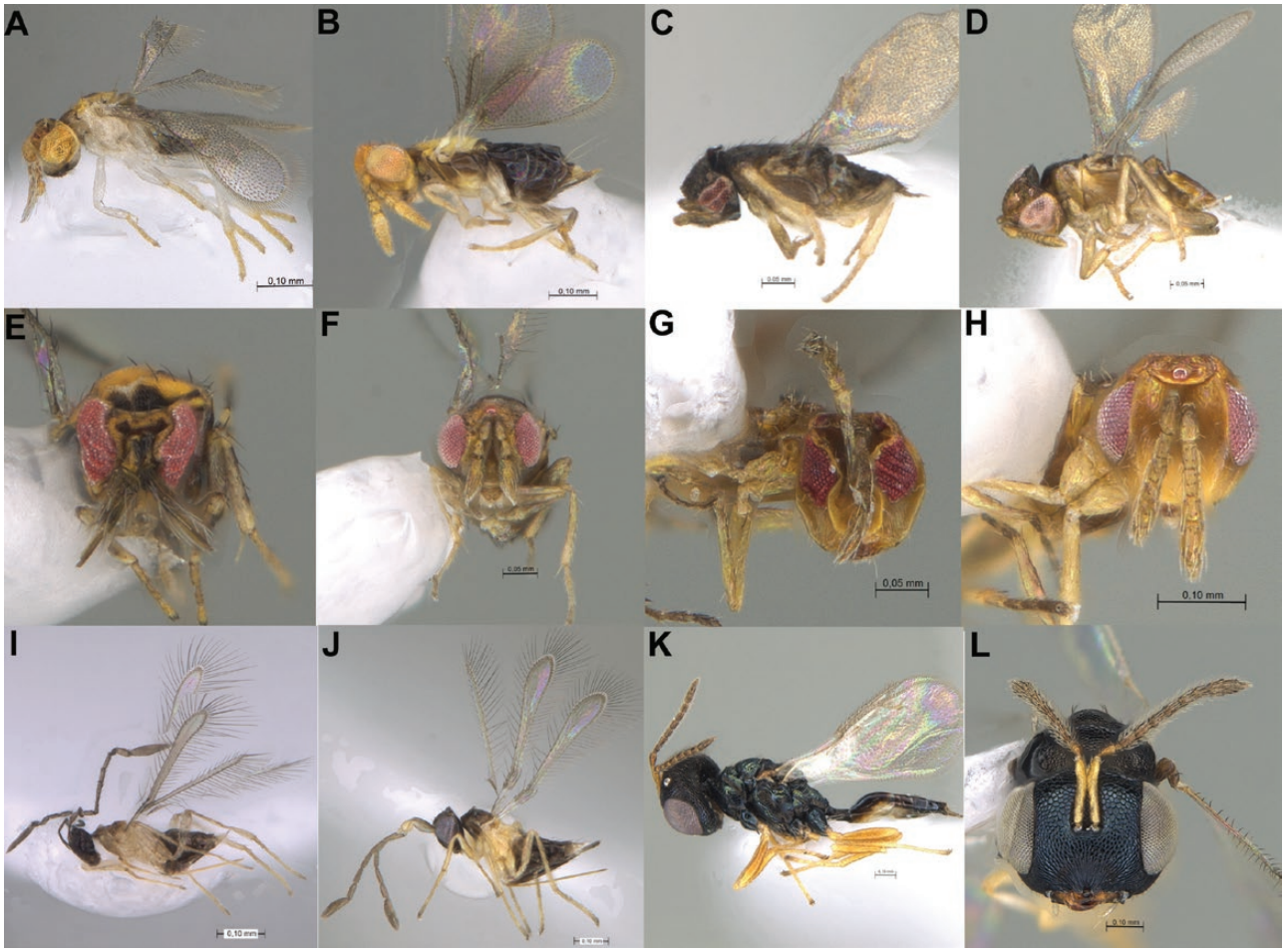

Fig. 2. Specimens of Chalcidoidea families under air drying from alcohol compared with treatments with Amyl acetate. A–B) Aphelinidae (T0 and T1), C–D) Encyrtidae (T0 and T2), E–F) Trichogrammatidae (T0 and T3), G–H) Eulophidae (T0 and T1), I–J) Mymaridae (T0 and T1), K–L) Pteromalidae (T4).

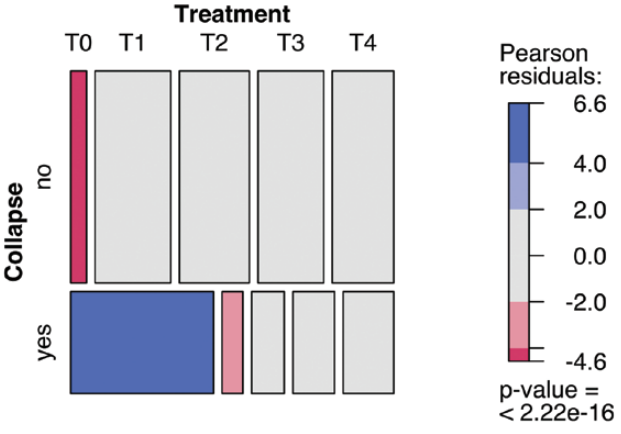

Fig. 3. Collapse independence model between evaluated treatments. Cell size is proportional to counts number. Colors represent level of residuals for that cell, blue means that there are more observations in that cell than would be expected under the test of independence and red means that there are fewer observations than would be expected under the same test.

Discussion

With amyl acetate, we achieved safe, fair, and faster results; dehydration of samples can take a couple of hours. Once time elapses, specimens can be removed from amyl with a brush and still be easily manipulated if any repositioning of appendages is desired before air-drying. As in AXA method (van Achterberg 2009), it is essential that not more than 20 min pass once extracted from the amyl solution to avoid losing an appendage or its head during pointing or carding because specimens are more fragile once dry and require correct handling.

Generally, in many Chalcidoidea, head (including antenna) and metasoma are most susceptible to collapse (Heraty and Hawks 1998). Still, in other taxa like pteromalid, head is well-sclerotized and does not collapse under any treatments, although metasoma wholly or partially collapses. Despite better results than air-drying from alcohol, some families like Eulophidae and Trichogrammatidae still present some problems regarding how they are affected by improper drying. However, even when CPD, HDMS, or Air Pump are used on soft-bodied specimens, generally some collapse or distortion of all body parts occurs (Heraty and Hawks 1998, Aguiar 2012). Therefore, it is necessary to continue developing or adjusting more drying techniques that allow better results on specific groups.

In addition to enhancing specimens' quality, it is also noteworthy that genomic DNA has been successfully extracted from amyl-acetate dried specimens such as Ichneumonidae, Encyrtidae (Austin and Dillon 1997), Braconidae (Quicke et al. 1999), and Pteromalidae (Maletti et al. 2021), insomuch as the process does not appear to cause DNA degradation or inhibit its subsequent extraction and PCR (Austin and Dillon 1997, Quicke et al. 1999), an

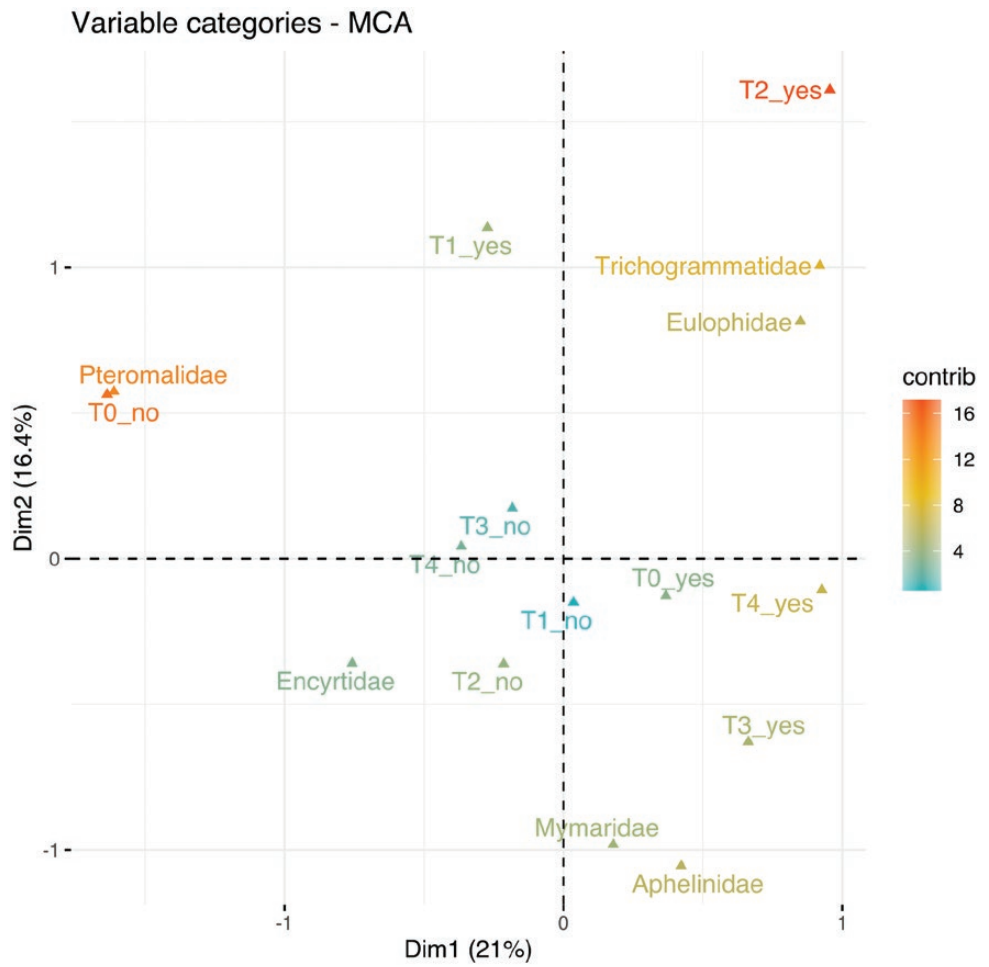

Fig. 4. Multiple correspondence analysis (MCA) between Chalcidoidea families (Hymenoptera) and their reaction to evaluated treatments. Variable categories that contribute most to dimension formation have been highlighted, in red with the highest contribution and in blue with the lowest contribution.

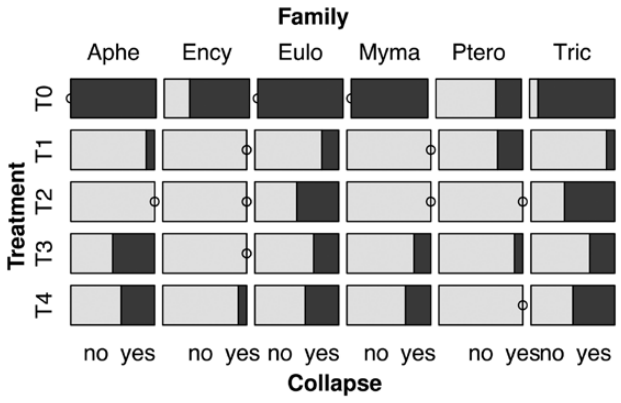

Fig. 5. Proportion between collapsed and not collapsed specimens by each combination resulting among groups of specimens per family and evaluated treatments. Apehlinidae (Aphe), Encyrtidae (Ency), Eulophidae (Eulo), Mymaridae (Myma), Pteromalidae (Ptero), Trichogrammatidae (Tric).

essential point for all and future molecular work that is being carried out on different taxonomic groups.

Although both reagents amyl acetate and HDMS need careful handling, the first one is considerably less harmful and dangerous to health and to the environment. Amyl acetate is a flammable liquid (category 3) and produces vapor, but it causes no harm if inhaled (see more here: www.sigmaaldrich.com/CO/en/sds/aldrich/s851981). HDMS is a highly flammable liquid (category 2), also producing vapor, and is harmful if swallowed or inhaled. HDMS contact is toxic to the skin, and harmful to aquatic life, with long-lasting effects (see more here: www.sigmaaldrich.com/CO/en/sds/aldrich/440191).

The lower cost of amyl acetate is an important factor. In general, the most recommended chemical would be HDMS but its cost is approximately $100.00 per 500 ml. While amyl acetate costs $2.50 per 500 ml, which represents a reduction of 97.5% of the total value of HMDS. Therefore, the use of amyl acetate entails a decrease in labor, and also requires less sophisticated, and costly equipment and reagents. Making this method feasible for small laboratories with limited resources.

Finally, various Chalcidoidea families of small size allow other mounting techniques to be used. One example is placing either temporarily or permanently such specimens on microscope slides to be examined under microscopy. Mounting specimens on slides reduces their exposure to ultraviolet radiation and protects them against physical disturbances. It is a specialized technique that requires more time, expertise, and the use of different reagents, including permanent mounting media, such as Canada balsam, which is four times the cost of HMDS and 97.5% costlier than amyl acetate. Furthermore, while all alternative preservation techniques should be considered, the result must allow complete examination of all angles to ensure that anatomical characters can be correctly observed,

which becomes difficult in the case of slide-mounted material as it configures a two-dimensional plane. This is why dry-mounted insects prevail as a basic and important technique that can facilitate morphological, taxonomic, and phylogenetic studies. Therefore, finding alternatives to improve these preexisting protocols continues to be important for conservation in entomological collections.

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Author Contributions

ALPB: Conceptualization-Equal, Formal analysis-Equal, Investigation-Lead, Methodology-Equal, Writing – original draft-Lead, Writing – review & editing-Equal. **EO-P:** Formal analysis-Equal, Investigation-Equal, Methodology-Equal, Writing – review & editing-Supporting. **JG:** Conceptualization-Equal, Formal analysis-Supporting, Project administration-Equal, Software-Supporting, Supervision-Supporting, Writing – review & editing-Equal. **EHD-B:** Conceptualization-Supporting, Formal analysis-Equal, Software-Lead, Writing – review & editing-Equal.

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