SLIDE-MOUNTING TECHNIQUES FOR *TRICHOGRAMMA* (TRICHOGRAMMATIDAE) AND OTHER MINUTE PARASITIC HYMENOPTERA[1]

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ABSTRACT: Methods for preparing Canada balsam slides of *Trichogramma* and similarly-sized parasitic Hymenoptera are presented. Included are procedures for transferring to balsam, specimens that were originally mounted in temporary, water soluble media, such as Hoyer's medium.

The primary factor responsible for our poor understanding of the systematics of minute parasitic Hymenoptera such as the Trichogrammatidae is the absence of adequate study collections. Certainly this can be attributed, in part, to difficulty in collecting which usually involves searching for hosts, or, more commonly, carefully sorting through bulk collections such as Malaise trap or sweep samples. However, once specimens are found the problem of preparing them for study perhaps is even more daunting. For detailed study most parasitic Hymenoptera with a body length of 1 mm or less must be placed on slides. This includes species of *Trichogramma* and those of virtually all other trichogrammatid genera. Unfortunately, there has been a tradition of mounting such wasps in water soluble media such as Hoyer's (e.g. Rosen and Debach 1979) or Liquid Faure (e.g. Doutt and Viggiani 1968) which, unless carefully monitored, eventually dehydrate and damage specimens (Upton 1993). The argument against using temporary media for small chalcidoids has been well made by Noyes (1982). Additional problems with earlier collections of micro-Hymenoptera are that specimens frequently were mounted uncleared and in a lateral rather than dorsoventral position, both practices precluding adequate examination of important characters, particularly the male genitalia, which in the Trichogrammatidae are important for classification and often identification as well (Nagarkatti and Nagaraja 1971, Viggiani 1971).

This paper presents the techniques developed over several years at the University of California, Riverside, primarily for preparing permanent Canada balsam mounts of *Trichogramma*. The methods also have been applied to other genera of Trichogrammatidae and are appropriate for other small, lightly-sclerotized wasps such as Aphelinidae. Because these groups are frequently placed in Hoyers we also describe our techniques for transferring such specimens to Canada balsam.

Publication of these methods is motivated by the large number of lots of *Trichogramma* received from biological control workers for identification and

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the frequent requests for mounting procedures. The continued popularity of this group for inundative biological control (Smith 1996) coupled with its taxonomic complexity (Pinto and Stouthamer 1994) requires authoritative species identifications. The chances of obtaining such identifications are enhanced if taxonomists are provided with slide-mounted specimens, as they generally have neither the time nor the resources for specimen preparation.

MATERIALS

The materials listed here are optimal for mounting lightly-to moderately-sclerotized small wasps the size of *Trichogramma* (< 1 mm).

a). Hooked probes. Useful for moving and orienting specimens. These are made using 0.10 mm & 0.20 mm diameter minuten pins. Pins are placed in the end of small wooden dowels and cemented in place with epoxy. The tips then are bent at right angles.

b). Chisel-tipped probes. These tools are useful for the optional removal of wings and antennae. They are made by using minuten pins (as above), hammering them into a flattened spatula (<1.0 mm wide) and then honing the edge on a very fine wet stone or emery paper.

c). Balsam applicators. Made with #1, nylon-headed, black insect pins inserted (head out) into wooden holders (old "00000" artist spotting brush handles work well). A hole is made in the end of the wooden holder with a #2 insect pin, the tip of the #1 pin is coated with epoxy cement and inserted into the holder.

d). Glass pipettes (12.5-15 cm), fitted with latex bulbs.

e). Forceps (fine-tipped jeweler's type); 2 pair.

f). Ceramic depression plate. Optimum size is ca. 9.0 x 11.5 cm with 12 depressions each 5.0 mm deep and 21 mm in diameter.

g). Rectangular plastic sealable food containers. Used for storing the depression plate during specimen clearing and dehydration.

h). Clear glass depression slide. Useful if specimens are to be dissected (see below).

i). Glass coverslips. 5 mm diameter, for specimen positioning. If difficult to obtain, 6 mm coverslips may be used instead. With the larger coverslips a ceramic plate with slightly larger depressions (item f) should be used. 6 mm diameter glass coverslips for specimen mounting. Small round coverslips are easier to use than the larger round or square varieties, require less mountant, and are less subject to entrapment of air bubbles. The 6 mm coverslips are available from D. J. and D. Henshaw; 34, Rounton Rd., Waltham Abbey; Essex, EN9 3AR; England. We currently are unaware of a source for the 5 mm coverslips.

j). Dehydrating, mounting and clearing solutions. Canada balsam (filtered-neutral); clove oil; ethyl alcohol dehydration series (10, 20, 40, 60, 80, 95, & 100% solutions); 10% potassium hydroxide (KOH) solution; Triton X-100®; xylene. The Triton X-100 is added to the 10% ethanol solution (6 drops/500 ml). The

Canada balsam is mixed in small batches (15-20 ml) with 15% clove oil by volume. This conditions the mountant and retards the rapid "skinning" of the balsam during mounting; it is critical for remounting specimens out of Hoyer's (see below).

MOUNTING PROCEDURE

The following method is recommended for dried specimens (either air dried or critical-point dried). It is followed by modifications necessary if working with live or alcohol-preserved specimens. Most of the procedures detailed below require a great deal of practice and patience before proficiency is attained. We suggest that preparators begin with expendable specimens.

Before mounting, it is important to have a comfortable work area and a stereo-microscope allowing magnifications up to ca. 100x. The work area under the microscope should have sufficient surface to accommodate the 9 x 11.5 cm depression plate through all of its movements, without the possibility of tipping or falling. Additionally, on either side of the magnifying field under the microscope and about 1 in. lower than the work surface, there should be room to support one's hands while making small, controlled movements during specimen positioning and mounting. Hand stability while manipulating specimens with the forceps or probes is critical. The microscope stage should be clear glass and elevated ca. 1 in. so light can be directed through the stage onto a white background to provide backlighting for the specimen. A twin-pipe, fiber optic illuminator with focusing tubes is an ideal light source. One pipe can be used for backlighting while the other provides direct illumination.

a). Preparation. Prepare a ceramic depression plate by adding two, 5 (or 6) mm coverslips and 80% ethanol to each depression. For this and all other procedures depressions should be filled to the top with liquids. Place one dry specimen in each depression and submerge in the ethanol. Using forceps, lift the coverslips and place the specimen beneath both. Place the plate in a container with a tight fitting lid and 95% ethanol in the bottom. The depression plate should be on a platform elevating it above the ethanol.

b). Clearing and softening. After ca. 12 hr, remove the 80% ethanol with a pipette and replace with 10% KOH (for this and all other exchanges liquid should be removed only to the level of the coverslip to insure that the specimen remains submerged). Sufficient clearing with KOH is important so that internal structures, particularly the male genitalia, will be visible. Most specimens can be left in KOH for 16-20 hrs at room temperature depending on the degree of sclerotization. This should be extended to ca. 30 hr for the most highly sclerotized specimens; less time is required for lightly sclerotized specimens. During clearing with KOH place the depression plate in a container with a tight fitting lid and distilled water in the bottom.

To accelerate clearing, specimens can be placed in a 30-40 C. warming oven for 1.0-4.0 hrs, again, depending on the degree of sclerotization. 1.0-2.0 hrs is

sufficient for most *Trichogramma*. After the first hour, warmed material should be checked every 30 min to prevent overclearing. Experience eventually allows one to estimate clearing time based on degree of sclerotization.

It is important that the specimen be under the two coverslips when the KOH is added. Otherwise it will float and is difficult to resubmerge. If floating occurs, resubmerging can be accomplished by lifting the coverslips with forceps and placing them above the specimen and gently coaxing it down to the bottom of the depression plate. If air bubbles become attached to the specimen during this process the KOH can be removed and replaced with 80% ethanol. This allows repositioning under the coverslips, while dissipating the air bubbles. The ethanol is then again replaced with KOH.

c). Specimen positioning. After material has cleared adequately, the KOH is removed with a pipette and replaced with 10% ethanol (with Triton X-100®). The specimen now is ready for positioning. Occasionally (particularly in *Trichogramma*) specimens will be adequately cleared except for gut contents in the abdominal area. When this occurs, the positioning should be carried out in KOH before the ethanol is substituted, thus allowing the KOH to penetrate the abdomen and complete the clearing process. This is especially important in taxa where an unhindered view of the genitalia is critical.

Positioning is the most critical and difficult step in the mounting process; it insures that the body and, most importantly, the genitalia are in the proper position for study. To a large extent, this step dictates the quality of the final preparation. The positioned specimen should end up somewhat dorsoventrally flattened, with dorsum adjacent to the coverslip, the legs and wings at roughly right angles to the body, and the antennae directed forward or laterally. The procedure used is as follows:

With two pair of forceps, move the uppermost coverslip to the side of the depression while holding the other in place over the specimen. Next, lift the second coverslip to free the specimen beneath. Hold the coverslip in a tilted position with the lower leg of the forceps resting on the bottom of the depression plate. The coverslip held in this position can be manipulated like the hinged lid of a box. With the coverslip in a slightly tilted position begin backing the specimen under its edge with the second pair of forceps, abdomen first and dorsum up (Fig. 1). As the specimen is pushed under, apply downward pressure with the coverslip at intervals to assure a slight dorsoventral flattening. The ability to manipulate the coverslip depends on adequate and comfortable hand support adjacent to the work area. The specimen can be moved under the coverslip by pushing on the front of the head with the tip of the closed forceps. Ideally, the legs and antennae move laterally during this process. As the specimen is backed under it is important to keep it in as perfect a dorsoventral plane as possible. This is especially important for males to provide optimum viewing of the genitalia. If adequately cleared, the genital capsule itself will be visible during positioning and slight adjustments can be made as necessary. The fore-

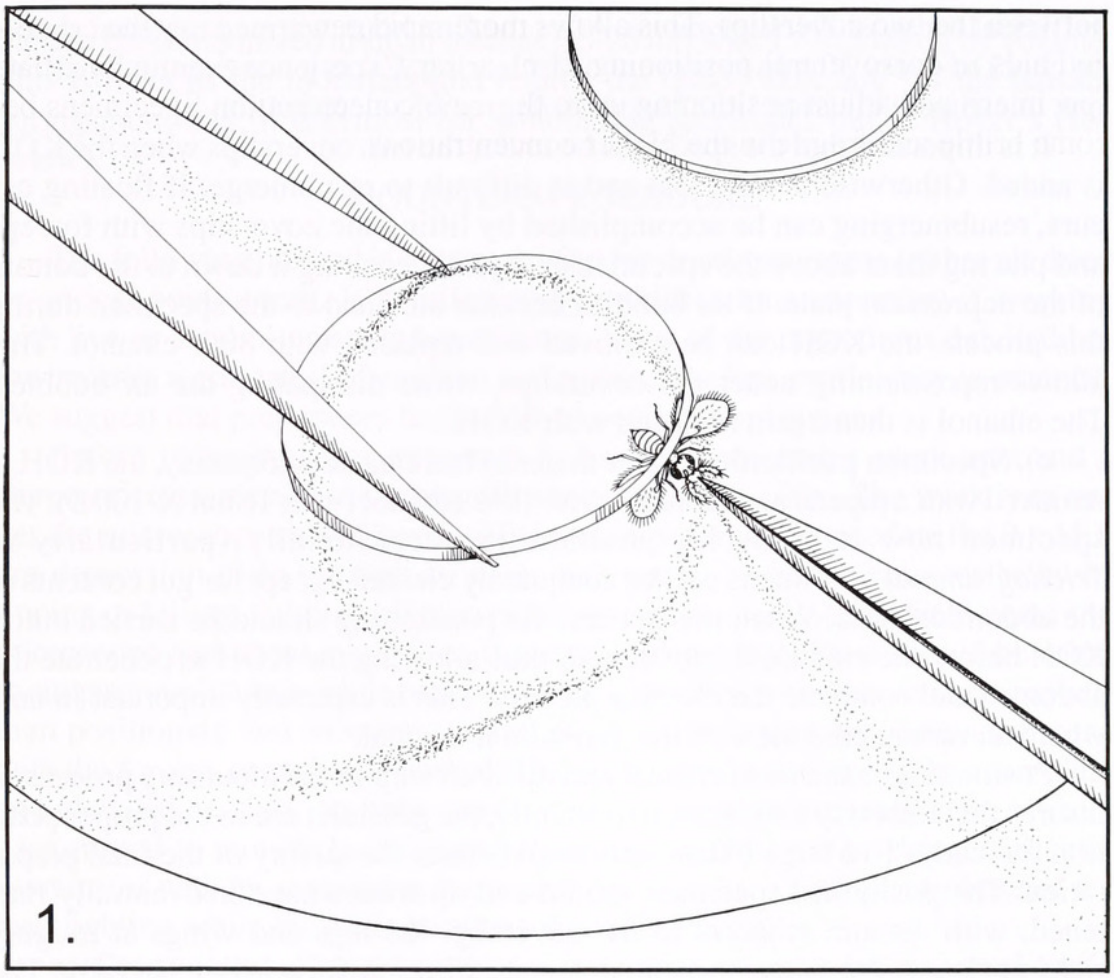

Figure 1. Positioning of a *Trichogramma* specimen (dorsum up) under a coverslip prior to dehydration and mounting (tilt of coverslip exaggerated).

wings normally reverse during this procedure, but this does not require correction because the location of the venation along the anterior border indicates that reversal has taken place. After positioning, the second coverslip is replaced on top of the first.

When working with dried individuals the legs sometimes extend directly below the body making it impossible to position specimens dorsoventally before movement under the coverslip. Such specimens, lying on their side, can be pushed, dorsum first, against the tilted coverslip and rolled into the correct position as the legs fold beneath the body. The specimen can then be pushed under as slight pressure is applied at intervals with the coverslip as indicated above.

d). Dehydration. After positioning is complete in 10% ethanol or KOH, the specimen is run through a series of higher ethanol concentrations (20, 40, 60, 80, & 95%) to absolute, keeping it at each concentration for 30-45 min. After the change to 40% the specimen should be repositioned from **under** to

between the two coverslips. This allows more rapid penetration by the ethanol and aids in dorsoventral positioning. If necessary, it is possible to manipulate specimens and adjust positioning up to the 60% concentration. Specimens become brittle and fragile in the higher concentrations.

Depression plates should remain in a tightly closed container during both the clearing and dehydration processes to prevent evaporation of liquids in the cells. During clearing and dehydration in 10 and 20% ethanol, the container should have distilled water in the bottom; 95% ethanol is used for the higher alcohol concentrations. The plates can be kept sealed for extended periods without liquid loss except at the 95% and absolute ethanol changes. At these levels the alcohol evaporates and specimens can dry out and be damaged if left for more than 1 hr.

e). Transfer to clove oil. After completing dehydration in absolute ethanol, the top coverslip is lifted, and the specimen is removed with a hooked probe (carefully hooking a wing or both antennae), gently transferred to clove oil and submerged. Material should remain in clove oil for at least 2 hr and can be stored for as long as 2 wks before mounting if kept in a closed, dust free container.

f). Modifications for ethanol-stored or live specimens. Excellent slides can be produced with dried material. However, live material or specimens stored in 70-80% ethanol can also be prepared directly for mounting. For ethanol-stored specimens, place individuals directly into 10% KOH for clearing. The procedure then follows that for dried specimens. If working with cultures or collecting parasitized eggs in the field, it may be desirable to prepare slides directly from live material. In this case, specimens should be killed once they are fully sclerotized by placing them in 75-80% ethanol for 12-24 hrs, and then treated the same as ethanol-stored material.

g). Mounting. Specimens can be mounted on slides either whole or after dissection. We find that *Trichogramma* and other relatively small trichogrammatids ($\leq$ 0.60 mm) are conveniently mounted whole. For larger specimens body depth may prevent the antennae and wings from being in an even plane for optimum viewing or photography. For these we routinely remove the antenna and wings from one side of the body, and mount them under a second coverslip with considerably less balsam. Some may prefer to dissect the head, both antennae, all wings and genitalia from the body, and mount them separately on the same slide. We do not do this because of time constraints. Furthermore, we find that mounting the male genitalia separately frequently distorts them and that they best retain their shape if left within the body.

1). Whole mounts. Before mounting, prepare a template to assist in placing the specimen at the center of the slide. Next, using the balsam applicator, pick up a small drop of mountant, dip it once in xylene, and gently "mix" it at the desired position on a clean slide. A dot of mountant ca. 3-4 mm in diameter is optimum. The specimen now can be placed in the mountant. Remove it from clove oil with the hooked probe and submerge it, dorsum up, in the balsam drop.

At this point the specimen can be oriented and some minor repositioning of body parts is possible. If the balsam is too tacky, a small drop of xylene can be added. After the balsam dries for 25-45 min (preferably covered to protect from dust), a coverslip (precleaned with 80% ethanol and lens tissue) is prepared. While holding the coverslip with forceps use the balsam applicator to lift another small dot of balsam, then dip the applicator in xylene and "mix" on the coverslip. Dip the applicator a second time in xylene and complete "mixing" before the coverslip is placed on the specimen. The area covered by the balsam dot should be ca. half that of the coverslip. The coverslip then is placed immediately on the specimen. For placement, it should approach the specimen from behind and at an angle, lowering it until the lower edge of the "coverslip balsam" contacts the "slide balsam". At this point the coverslip is released and allowed to settle. The coverslip and specimen can be manipulated slightly by gently touching and moving the coverslip with forceps during or immediately after settling.

It is important that the correct amount of balsam be used, which, of course, depends somewhat on the size of the specimen. Too little may crush it as the slide dries; too much usually results in too thick a mount and body parts settling off horizontal, precluding proper focusing or measuring.

2). Dissected specimens. We typically mount the right antenna, and the fore and hind wings from the same side of the body under one coverslip above the specimen they are taken from. For dissection, transfer the specimen from clove oil to a clear glass depression slide to which one drop of clove oil has been added. It is best to have backlighting for this procedure as it allows optimum tracking of dissected parts, which are difficult to see when cleared. Using a hooked probe and chisel-tipped probe, orient the specimen dorsum up with the head directed toward or away from you, depending on the side to be dissected. To remove the forewing, pin it at its base against the bottom of the depression slide with the chisel-tipped probe and gently move the specimen away with the hooked probe. The same procedure is used for the hind wing and the antenna. After removal from the body, the structures are stacked upon one another (forewing at bottom) and transferred with a hooked probe to a balsam dot (1.0 - 2.0 mm in diam.) on the slide. Once submerged in the balsam, the structures are separated and oriented. Wing orientation should be the same as if still attached to the body. The body with parts still attached is mounted under a separate coverslip below. Preparation of balsam dots and coverslips are the same as indicated for whole mounts.

Some taxa are deep bodied and difficult to keep in a dorsoventral plane after mounting. For these it may be necessary to add pieces of broken coverslip to either side of the specimen or to add additional balsam in layers over a period of time.

h). Drying. Completed slides must be kept flat until the balsam sets. They can be placed either in a slide folder for 1-2 wks, or on a tray in a drying oven at 30-40 C for 3-5 da.

REMOUNTING PROCEDURE

This procedure allows specimens originally mounted in Hoyer's medium to be transferred to Canada balsam with minimal damage. A significant problem in remounting is that the antennae and head frequently collapse after contacting the balsam. The following method, while not completely successful, prevents major collapsing in 80-95% of the specimens. The avoidance of xylene is the primary difference from the previous procedure.

a). Coverslips of Hoyer's mounts frequently have been sealed with various compounds to reduce desiccation. This material should be removed with the tip of a razor blade or Exacto® knife before processing.

b). Place slide in a Petri dish and soak in distilled water for ca. 60 hrs. After soaking, the coverslip can be lifted free of the specimen(s).

c). Transfer specimen with a hooked probe to a ceramic depression plate supplied with 10% ethanol (and Triton X-100®). Cover it with a 6 mm coverslip.

d). Replace ethanol with 10% KOH for 30-40 min at room temperature. KOH not only is a clearing and softening agent but also reduces head and antennal collapse. Consequently, specimens should be treated with KOH even if they had previously been cleared for the Hoyer's mount. Because of flattening from the previous mount it is very difficult to reposition specimens during the remounting process.

e). Remove the KOH with a pipette and replace with 10% ethanol for 30 min. Repeat with 20%, 40%, 60%, 80%, 95%, and twice with 100% ethanol. If necessary, specimens can be held longer (e.g. overnight) at concentrations of 80% or lower.

f). Replace the absolute ethanol with a 1:1 mixture of absolute ethanol and clove oil. Place the depression plate into a partly opened container for 2-3 wks to allow for slow, complete evaporation of the alcohol and any remaining water. The presence of the smallest amount of ethanol or water at this point results in partial or complete collapsing of the antennae and head during mounting. Once evaporation is complete only clove oil will remain and the specimen is ready for mounting.

g). The mounting procedure is similar to that used for new material but with the following differences. i) Xylene should never be used; using it at this stage results in structure collapse. If the balsam (with 15% clove oil) becomes too tacky, a drop or two of additional clove oil can be added. ii) The balsam dot placed on the slide should not be mixed or spread significantly, nor should it be allowed to dry after specimen placement. iii) Coverslips are placed immediately after specimen placement and are dry (i.e. no balsam is added to the coverslip first). iv) When placed on the specimen, the coverslip is not tilted but released parallel with the slide. This allows it to contact the balsam drop near its center and forces air bubbles out when pressure is applied with the forceps. v) Speci-

men transfer should be made as quickly as possible. Delays at this point can result in the balsam losing its working consistency and will damage the specimen.

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LITERATURE CITED

Doutt, R. L. and G. Viggiani. 1968. The classification of the Trichogrammatidae (Hymenoptera: Chalcidoidea). Proc. Calif. Acad. Sci. (4th ser.) 35: 477-586.

Nagarkatti, S. and H. Nagaraja. 1971. Redescriptions of some known species of *Trichogramma* (Hym., Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. Bull. Entomol. Res. 61: 13-31.

Noyes, J. S. 1982. Collecting and preserving chalcid wasps (Hymenoptera: Chalcidoidea). J. Nat. Hist. 16: 315-334.

Pinto, J. D. and R. Stouthamer. 1994. Systematics of the Trichogrammatidae with emphasis on *Trichogramma*. In: Biological control with egg parasitoids, E. Wainberg and S. A. Hassan (eds.), Wallingford (UK), 1-28.

Rosen, D. and P. DeBach. 1979. Species of *Aphytis* of the world (Hymenoptera: Aphelinidae). Ser. Entomol. 17, 801 pp. (W. Junk BV, The Hague).

Smith, S. M. 1996. Biological control with *Trichogramma*: Advances, successes, and potential of their use. Annu. Rev. Entomol. 41: 375-406.

Upton, M. S. 1993. Aqueous gum-chloral slide mounting media: an historical review. Bull. Entomol. Res. 83: 267-274.

Viggiani, G. 1971. Ricerche sugli Hymenoptera Chalcidoidea XXVIII. Studio morfologico comparativo dell'armatura genitale esterna maschile dei Trichogrammatidae. Boll. Lab. Entomol. Agr. 'Filippo Silvestri' di Portici 29: 181-222.