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A PROTOCOL TO PRESERVE THE INTEGRITY OF STABLE FLY (DIPTERA: MUSCIDAE) DNA FOR LONG DISTANCE SHIPMENT

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ABSTRACT

Population genetic studies on a global scale may be hampered by the ability to acquire quality samples from distant countries. Preservation methods must be adequate to prevent samples from decay during shipping, so an adequate quantity of quality DNA can be extracted for analysis, and materials used must not be prohibited by the pertinent Postal Service. We tested a shipping protocol for stable flies which preserves the samples and adheres to Postal regulations. Samples were soaked in 95% ethanol for 6, 12, 24 and 48 hours; the ethanol was then poured off. Samples remained 'dry' for 2, 4, 6 and 8 days to simulate time in transit; ethanol was replaced at the end of each time point. Soaking for 6 hours produced the most DNA when samples were in transit for 2-4 days, but DNA quantity declined abruptly after 4 days. Soaking for 24 hours was more effective than soaking for 12 or 48 hours and was the best protocol for 6-8 days in transit. Results show that samples collected from distant locations (8 days in transit) should be imbibed in 95% ethanol for 24 hours before shipping, but for short distances (< 4 days in transit) samples imbibed for 6 hours produce quality DNA.

Keywords: Biological sample transport, Preservation methods, *Stomoxys calcitrans*

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L.), is a haematophagous pest of livestock, pets and humans which is distributed worldwide. Its parasitism of livestock causes a decrease in weight gain and milk production due to blood loss, stress and avoidance behaviors (Campbell *et al*., 2001; Catangui *et al*., 1993), with economic losses estimated at > $1 billion annually in the United States (Taylor and Berkebile, 2006). Therefore, more efficient control methods of this insect are continuously being sought. Genetic studies are at the forefront, with population genetics being of primary interest due to the dynamics of stable fly behavior. Stable flies are known to fly long distances and disperse with the wind (Fye *et al*., 1980; Hogsette *et al*., 1989), which suggests the possibility of gene flow between distant areas. Population genetic studies have been conducted at the local and regional level to determine the origin of stable fly outbreaks, immigration vs. overwintering, and genetic variation within regions (Szalanski *et al*., 1996; Taylor *et al*., 2007; Oliviera *et al*., 2008; Kneeland *et al*., 2013). Recently, global population studies have been conducted to examine the evolutionary origins of stable flies and the global population differentiation (Marquez *et al*., 2007; Dsouli-Aymes *et al*., 2011; Kneeland, 2011).

Successful genetic research requires the acquisition of samples containing quality DNA. Various sample collection methods are used for stable flies, including sweep nets, traps and sticky cards (Taylor and Berkebile, 2006). Good quality DNA can be obtained using these methods if the samples are preserved soon after collection. Preservation methods include freezing at -80ºC, lyophilization (freeze drying) and soaking in 95% ethanol. If collections are made at a local scale, any of these methods are efficacious and the DNA will remain undamaged. However, global population studies require acquiring samples from distant countries and shipping regulations put restraints on the methods that can be used (Clark *et al*., 2009).

Storing samples at -80ºC is a very effective preservation method, but not for samples to be shipped long distances. If put on dry ice, the samples would remain cold for some time, but sublimation may occur before arrival at the destination.

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Additionally, it is not permissible to ship dry ice internationally through the postal system, which would eliminate this method for global studies.

Lyophilization is effective and dried samples can be shipped through the mail. Lyophilization is commonly used for plant tissue and bacterial samples; samples preserved with this method yield large amounts of DNA. However, it is an expensive process and many locations from which samples are desired would not have access to the equipment needed for lyophilization (Clark *et al*., 2009).

Soaking in 95% ethanol is an inexpensive, efficient method of preservation. Small vials of ethanol can be carried into the field and samples preserved immediately after collection, eliminating the possibility of decomposition beginning between collection and deposition in a -80ºC freezer. It is prohibited to ship ethanol by air transportation in amounts >30 ml, which may preclude sending adequate sample sizes (Bonin *et al*., 2007), through the postal service (National Park Service, 2007). A method was developed and described herein where specimens were preserved in ethanol before shipping, ethanol was removed for shipping and then replaced on reaching the destination laboratory and the DNA quality and quantity was retained.

MATERIALS AND METHODS

Experimental design

Adult stable flies were obtained from a laboratory colony. All individuals were from the same cage, eliminating genetic differences due to population variation and condition of the samples and were placed in 15 ml tubes containing 95% ethanol immediately after collection. A time point experiment was developed to simulate shipping conditions. The variables were the time that the adults were allowed to soak in 95% ethanol (6, 12, 24 and 48 h) and time between removal and replacement of ethanol to simulate shipping times (2, 4, 6 and 8 days). This experiment was replicated 4 times. Ten stable flies were put into each tube, and 5 of the 10 were randomly chosen for DNA extraction.

DNA extraction

DNA was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method modified from Doyle and Doyle (1987). Samples were first washed in autoclaved double distilled water for 10 minutes. The head, abdomen, wings and legs were detached from the thorax, and the gut was removed. The thorax was homogenized in 250µl CTAB buffer (100 mM Tris-HCl, 1.4M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% β-mercapto ethanol) in a 1.5ml microcentrifuge tube, then an additional 250µl CTAB was added. Fifteen microliters of RNaseA was added to each tube and tubes were incubated on heat blocks for 2h at 65ºC. Samples were mixed every 20 min by inversion of the tubes. Proteinase K (10µl) was added to each tube and they were incubated at 37ºC for 1h.

Samples were then centrifuged for 5 min. at 14,000 rpm and 20ºC to separate tissue from supernatant. The supernatant was transferred to a new, autoclaved 1.5ml tube, chloroform: isoamyl alcohol (24:1; 500 µl) was added to each sample and each was centrifuged at 14,000 rpm and 20ºC for 20 min. The top, aqueous layer was transferred to a new, autoclaved 1.5ml tube. The chloroform:isoamyl step was repeated; the top, aqueous phase was transferred to a new tube and 400 ml chilled (-20ºC) isopropanol was added to precipitate the DNA. Samples were stored at 4ºC over night.

Samples were centrifuged for 30 min. at 14,000 rpm and 4ºC, at which time a white pellet of DNA formed at the bottom of the tube. The isopropanol was poured off; samples were washed with chilled absolute ethanol, centrifuged for 5 minutes at 14,000 rpm and 4ºC and the ethanol removed. The ethanol wash was repeated using 70% ethanol, the ethanol was completely removed and the samples were allowed to air dry. After drying, the DNA was re-suspended in 50µl 1X TE buffer and stored at -80ºC.

Data collection

The amount of DNA (ng/µl) was determined from readings with the Nanodrop® spectrophotometer (ThermoScientific) and used as data. Each of the 5 sub-samples (individual flies) from all tubes (1 soak time and 4 shipping times) were analyzed for quantity of DNA extracted; 3 readings were taken from each sub-sample. The pedestals of the Nanodrop® were cleaned after each reading with a dry tissue. After each group of 20 flies, the pedestal of the spectrophotometer was cleaned with nanopure H_2O and a new blank was applied. Nanodrop® readings were on 1.5µl of DNA; if the DNA sample did not form a bead on the pedestal, the pedestal was cleaned and the sample was re-loaded.

Statistical analysis

Data were analyzed using the PROC MIXED procedure (SAS Institute, 2009). One data point was missing, so 959 of 960 data points were analyzed as a Randomized Complete Block Design of a 4 x 4 factorial with subsamples; the replications were the blocks, hours soaked and days after pour-off were fixed factors, and the subsamples were the 5 individual flies in each hour/day combination.

RESULTS

There were no interaction effects between hours and days (Table 1) so the Differences of Least Square Means was used to detect differences between the hours soaked in 95% ethanol averaged over the days and the differences in time after pour-off averaged over the hours soaked. The samples soaked for 6 h contained a higher amount of DNA than 12, 24 or 48 h at the 2d and 4d points, but dropped off considerably at 6 and 8 d (Fig. 1). The 24h samples produced higher amounts of DNA than 12h and 48h at all days, and more than 6h at 6d and 8d, while the 48h samples produced the least amount of DNA at all days (Fig. 1). The amount of DNA produced by the 6h and 24h samples was significantly ($P = 0.0051$ and $P = 0.0035$ respectively) higher than those soaked 48h (Fig. 1). The samples left for 2d after pour-off produced significantly more DNA than those left for 6d and 8d ($P = 0.0152$ and $P = 0.0004$ respectively) and the samples left for 4d after pour-off produced significantly more DNA ($P = 0.0325$) than those left for 8d (Fig. 1).

DISCUSSION

Soaking stable flies in 95% ethanol for 24h before pour-off consistently produced more DNA than soaking for 12h or 48h and 24h soaking produced more DNA than the 6h samples at 6d and 8d. However, within the United States or over short distances, where shipping can be expedited, soaking for 6h would be sufficient, because the 6h samples produced the highest amount of DNA at 2d and 4d.

It was unexpected that the 48h samples yielded the least amount of DNA; it was assumed that soaking for a longer period of time would preserve the samples more thoroughly. This experiment could be replicated to determine if other factors were influencing the 6h and 48h results and to test longer periods after pour-off, such as 14 or 21 days. The condition of the samples collected, before placing in ethanol (i.e. covered in adhesive from sticky traps), could also be considered in a similar experiment. In this test, all flies were collected from the same rearing cage and killed immediately in 95% ethanol. However, samples collected in the field may not be handled in the same manner. If they are collected from traps or sticky cards they could be dead before collection and may not be imbibed in ethanol until return to the laboratory, or longer. It would be more representative of field collections to design a similar experiment using collection method and time after collection before soak in addition to the factors used herein.

CONCLUSION

A protocol was developed for shipping stable fly samples long distances while retaining the quality of the DNA. Samples are placed in a vial of 95% ethanol immediately after collection, or as soon as possible thereafter. The samples are imbibed in the ethanol for 24 h after which the ethanol is poured off and a cotton ball is inserted into the vial before sealing. The vials are shipped, ethanol is added to the vials upon arrival at the destination laboratory and high quantities of quality DNA can be extracted from the stable flies. This protocol has been successfully applied (Kneeland, 2011; Kneeland *et al*., 2015) and may also be applicable for shipping other species of insects from distant sample locations for use in molecular genetic analyses.

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Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. USDA is an equal opportunity provider and employer.

Table 1

The Type 3 Test of Fixed Effects generated by SAS indicates no significant interaction
between hours and days (P=0.3086), so the Difference of Least Square Means could be used to
analyze the differences between hours soaked in 95% ethanol and time after pour-off.

Type 3 Tests of Fixed Effects

Effect	Num Df	Den Df	F Value	Pr > F
Hour	3	45	4.35	0.0089
Day	3	45	5.20	0.0036
Hour*Day	9	45	1.22	0.3086

No hour*day interaction: P = 0.3086.

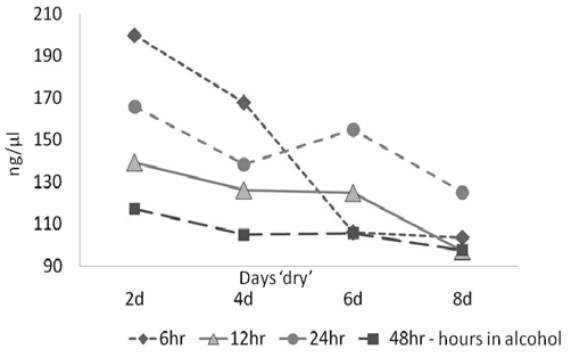

Fig. 1

Plot of hours soaked in 95% ethanol (lines), days after pour-off (x-axis; day 'dry'), and amount of DNA (ng/µl) (y-axis). The graph indicates no significant interaction between hours soaked and days after pour-off. The amount of DNA (ng/µl) reduces dramatically in the 6h samples after 4 days.

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