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Comparison of Preservation Methods of *Atta* spp. (Hymenoptera: Formicidae) for RAPD Analysis

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Comparação de Métodos de Preservação de *Atta* spp. (Hymenoptera: Formicidae) para Estudos com RAPD

RESUMO - DNA de alta qualidade para estudos moleculares pode ser facilmente extraído de espécimes frescos. Entretanto, amostras frescas são difíceis de ser mantidas vivas por longos períodos tornando a sua preservação um sério problema, principalmente quando são coletadas e transportadas de locais distantes. Com o objetivo de estabelecer um método eficiente de preservação de *Atta* spp. (formigas cortadeiras) para extração de DNA visando análises de RAPD, seis diferentes métodos de armazenamento foram avaliados: 1) –70ºC; 2) etanol 95% a –20ºC; 3) etanol 95% a 4ºC; 4) etanol 95% à temperatura ambiente; 5) sílica gel à temperatura ambiente; e 6) tampão (0,25 M EDTA, 2,5% SDS, 0,5 M Tris-HCl, pH 9,2) à temperatura ambiente. O DNA foi extraído (Cheung *et al*., 1993 - modificado) e examinado aos 90, 210 e 360 dias após o armazenamento. Espécimes frescos foram usados como controle. O DNA total foi medido com minifluorímetro. A qualidade do DNA foi determinada escaneando-se as fotos com densitômetro e a integral da varredura foi calculada para DNA ≥ 9,4 kb. Os dados foram analisados usando o delineamento estatístico de blocos inteiramente casualizados com parcelas subdivididas e com quatro repetições. Todos os métodos foram eficientes para preservar *Atta* spp. até 210 dias. Até 360 dias, o DNA foi degradado somente em etanol 95% à temperatura ambiente, resultando na ausência de bandas no perfil RAPD quando comparado com os demais métodos de presevação. Embora a preservação a baixas temperaturas seja recomendada para longos períodos, o armazenamento em sílica gel e tampão pode ser considerado alternativa satisfatória quando refrigeração e transporte são fatores limitantes.

PALAVRAS-CHAVE: Insecta, formiga saúva, condições de armazenamento, extração de DNA.

ABSTRACT - High quality DNA for molecular studies can be easily extracted from fresh specimens. However, live samples are difficult to keep for long periods thus making their preservation a serious problem, specially when they are collected and transported from remote locations. In order to establish an effi-

cient method to preserve *Atta* spp. (leaf-cutting ants) for RAPD analysis, six different storage methods were examined: 1) -70ºC; 2) 95% ethanol at -20ºC; 3) 95% ethanol at 4ºC; 4) 95% ethanol at room temperature; 5) silica gel at room temperature; and 6) buffer (0.25 M EDTA, 2.5% SDS, 0.5 M Tris-HCl, pH 9.2) at room temperature. DNA was extracted (Cheung *et al*., 1993 - modified) and examined after 90, 210 and 360 days of storage. Freshly killed specimens were used as control. DNA yield was measured with a minifluorometer. DNA quality was determined by scanning photographs with a densitometer and the integral of the scan was calculated for DNA of size ≥ 9.4 kb. Data were analyzed using a completely randomized split-plot design with four replicates. All methods were efficient to preserve *Atta* spp. DNA up to 210 days. At 360 days, DNA was degraded only in 95% ethanol at room temperature, which resulted in RAPD profiles with missing bands. Although preservation at low temperatures is recommended for long periods, methods using silica gel and buffer can be considered satisfactory alternatives when refrigeration and transportation are limiting factors.

KEY WORDS: Insecta, leaf-cutting ants, DNA extraction, storage conditions.

The development of new techniques in molecular genetics, such as polymerase chain reaction (PCR), has revolutionized studies on molecular biology of insects. However, in many molecular analysis, the limiting factor is the difficulty in obtaining DNA from live material. Generally, DNA has to be extracted from specimens stored in different killing/preservative agents and at varied temperature conditions. Since the majority of the techniques used in molecular analysis require DNA of good quality with minimum degradation, special consideration should be given to sample preservation. Occasionally, difficulties to maintain the insects alive for long periods, problems with importation and labor capacity in the laboratory require storage techniques that allow the use of simple DNA extraction methods without significant losses in yield and quality.

There have been several reports to determine the effects of different storage conditions on the quality of extracted DNA, mainly on samples from vertebrate tissues (Goelz *et al*. 1985, Smith *et al*. 1987, Arctander 1988, Bramwell & Burns 1988), but these results are not necessarily extrapolated to insects. Specifically for insect preservation, a number of variables, such as reagents concentration, temperature, species and age of the specimens have been reported to alter the amount and/or quality of the DNA, and consequently the pattern of bands obtained in RAPD (Post *et al*. 1993, Reiss *et al*. 1995, Dillon *et al*. 1996, Koch *et al*. 1998). The killing agent is one of the factors that can have a strong influence on the quantity and quality of the DNA extracted from preserved insects. Insects killed with ethyl acetate vapour (a product commonly used by entomologists) and subsequently dried at room temperature, yielded small amounts of DNA with unsatisfactory quality for PCR analysis. The use of ethyl acetate to kill insects has been pointed out as the reason why many studies with museum insects have failed to extract good quality DNA and produce consistent results (Dillon *et al*. 1996).

Preservation of insects in ethanol is a standard practice, but the possibility of improper use of this preservative and some restrictions occasionally applied to its transportation have led to the search for alternative methods for insect preservation methods. Liq-

uid nitrogen is also considered a standard technique to preserve insects, but its high cost and handling difficulties make this method unsuitable for field collections. Except for liquid nitrogen, with most of the preservative agents such as ethanol, methanol, paraformaldehyde solutions or saline solutions containing cation-chelating agents (such as EDTA), the degree of DNA degradation may vary largely depending on the origin of the sample (Laulier *et al*. 1995).

Although effects of various preservation methods on DNA quality have been reported for different insect species, it is difficult to extrapolate results from one group of insects to another. Considering that the species is one of the factors that can affect the quality and amount of the DNA isolated from preserved insects, the objective of this study was to select preservation methods for *Atta* spp. that allow the recovery of large quantity of high-molecular weight DNA suitable for RAPD analysis.

Material and Methods

Six different preservation treatments, at controlled and room temperatures (RT), were compared: (1) -70ºC; (2) 95% ethanol at -20ºC); (3) 95% ethanol at 4ºC; (4) 95% ethanol at RT; (5) silica gel at RT; (6) buffer solution (0.25 M EDTA, 2.5% SDS, 0.5 M Tris-HCl, pH 9.2) at RT. DNA extracted from fresh specimens was used as control. In all treatments, specimens were stored for 90, 210 and 360 days prior to DNA extraction.

Live maxima workers (soldiers) of *Atta sexdens rubropilosa* Forel were collected from only one nest in Assis-county, state of São Paulo, Brazil, in September 6, 1997. The specimens were placed in glass flasks containing 95% ethanol and brought to the laboratory. After 48 h, thirty individuals per treatment (ten per each storage time) were transferred to a dessicator under vacuum for 30 min. The insects were than placed in 50 ml glass vials, according to the different preservation treatments and the lengths of time between collection and DNA extraction, except for treatments 1 and 5 that were transferred to 1.5 ml microtubes and 200 ml glass flasks, respectively. In treatment 5, a disk of filter paper, with the same diameter of the flask, was placed between 2 cm silica gel bottom layer and the ants before closing the flasks with rubber seal caps and parafilm.

DNA was extracted only from the head of each ant to avoid the presence of foreign DNA, such as pathogens, endoparasites and ingested biological material. A modified version of the DNA extraction protocol of Cheung *et al*. (1993) was used to isolate total DNA. Before DNA extraction, dissected heads were placed in a dessicator under vacuum for 30 min and then crushed in a 1.5 ml microtube containing 200 µl extraction buffer (200 mM Tris-HCl, pH 8.0, 2 M NaCl and 70 mM EDTA), using an electric homogenizer and sterilized disposable plastic pistils. After that, 50 µl of 5% sarcosyl was added, homogenized again and incubated at 65ºC for 30 min. The solution was centrifuged (10000 g for 15 min) and the supernatant transferred to a fresh microtube. DNA was precipitated by adding 110 µl of 10 M ammonium acetate and 250 µl of cold isopropanol. The solution was left overnight at 4ºC, then sedimented by centrifugation for 15 min at 10000 g. The supernatant was discarded, the pellet was washed twice with 70% ethanol, air dried and resuspended in 50 µl of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) containing RNase 10 µg/ml), and stored at -20ºC. Total DNA obtained in each extraction was quantified by fluorescence with Hoechst 33258 dye using a DyNA Quant 200 minifluorimeter (Hoefer Instruments).

To determine the DNA quality, 1µg of DNA for each sample was analyzed, by gel electrophoresis at 5 V/cm in agarose 0.8% using 0.5X TBE (tris-borate-EDTA) as running buffer and stained with ethidium bromide. Gels were photographed under UV light with Polaroid 667 film using a Polaroid MP 4 Camera. The photographs were scanned using a densitometer (Bio-Rad, model 620) and the integral of each scan was calculated for DNA above 9.4 kb. The statistical analysis of

the scanning data was made using a completely randomized split-plot design with four replications, where the preservation methods were the main plots and storage periods the subplots.

To confirm that the DNA extracted from insects preserved in different conditions could be used for RAPD analysis, the reproducibility of the band pattern were evaluated using DNA extracted after 210 and 360 days of storage. Amplification reactions were performed in volumes of 25 µl containing 25 ng of template DNA. The reaction buffer consisted of 100 mM of each dNTP, 0.2 µM primer (OPB-01 and OPB-07, Operon Technologies, Inc.), 1.0 unit of *Taq* polymerase in 10 mM Tris-HCl (pH 9.0), 3.0 mM $MgCl_2$, 50 mM KCl and 0.05% BSA. Amplifications were performed in a PTC-100™ (MJ Research, Inc.) thermocycler programmed as follows: 3 min of denaturation at 94ºC, 3 min of annealing at 35ºC, 2 min of extension at 72ºC and 40 steps of 1 min at 94ºC, 1 min at 36ºC, 2 min at 72ºC, and after the last cycle a final extension of 72ºC for 5 min. Fragments generated by the amplification were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light as previously described.

Results and Discussion

Data on amount of DNA extracted from the heads of *A. sexdens rubropilosa* in the different preservation methods and storage periods are shown in Table 1.

Although the ANOVA indicates that the effects of the storage period and of the interaction between preservation method and storage period were not significant ($F = 28.15$, $P>0.05$), specimens preserved in 95% ethanol at RT consistently had lower yields of DNA in all three storage periods. This can be confirmed by comparing the treatments means, where preservation in 95% ethanol at RT differed significantly from the other preservation methods, having a detrimental effect upon DNA yields, specially after 360 days of storage (Table 1). The decrease in DNA recovery in 95% ethanol at RT after 210 days of storage, in relation to the yields obtained in 95% ethanol at 4ºC, is similar to the reduction in DNA recovery reported by Post *et al*. (1993) with females of *Simulium damnosum* s.l. (Diptera: Simuliidae) in 80% ethanol at RT when compared with 100% ethanol at 4ºC, after 120 days of preservation.

Similarly, Reiss *et al*. (1995) observed a significant decrease in the amount of DNA isolated from *Amara glacialis* (Mannerheim) (Coleoptera: Carabidae) stored in 95% ethanol at RT after only 73 days, leading to a 100-fold reduction after 144 days. Koch *et al*. (1998) also extracted low amounts of high molecular weight DNA, from heads of *Simulium vitattatum* Zetterstedt preserved in 80% ethanol at RT for approximately four years. The small quantity of extracted DNA was attributed to the small size of the insect's head. Although there is a clear indication that the storage time of insects in 95% ethanol at RT for DNA conservation is relatively short, in the specific case of *Atta* a significative reduction in DNA yield was not observed up to 210 days.

The effect of the temperature on the preservation of *Atta* DNA in ethanol can be observed comparing methods 2 (95% ethanol at -20ºC), 3 (95% ethanol at 4ºC) and 4 (95% ethanol at RT). The amount of DNA obtained was statistically similar at -20°C and 4°C. However when *Atta* was stored at room temperature a lower amount of DNA was recovered (Table 1). These findings are consistent with results obtained with preservation of *S. damnosum* in 95% ethanol at 4ºC (Post *et al*. 1993) in which only a small reduction in the amount of DNA was observed in insects preserved under such conditions, even after 371 days of storage. On the other hand, they reported poor yields of DNA when the insects were preserved in 95% ethanol at -20ºC, for the same period of storage, which is in dis-agreement with the results obtained in this study.

Considering the overall mean of the three storage periods, silica gel at RT was the preservation method that gave the largest amounts

Table 1. DNA yield (μg) extracted from heads of *A. sexdens rubropilosa* at 90, 210 and 360 days of storage under various conditions.

	Storage period (days)			Mean
Treatments	90	210	360	
-70ºC	3.9	4.2	3.6	3.9ab
Ethanol 95% at -20ºC	3.9	5.0	3.6	4.1ab
Ehtanol 95% at -4ºC	3.1	3.4	4.1	3.5 b
Ethanol 95% at RT	2.0	2.1	0.4	1.5 c
Silica gel at RT	4.8	4.4	4.8	4.7a
Buffer at RT	4.3	3.7	3.9	3.9ab
Fresh specimen	4.4	3.9	4.4	4.2ab
Mean	3.8	3.8	3.5	

Means followed by the same letter are not significantly different by Tukey's Test at 5% level of significance.

of DNA. Excellent DNA yields were also obtained by Post *et al*. (1993) with this preservation method. These results are probably due to the fact that the insects were quite dehydrated and brittle after all three storage periods, which allowed a better grinding and homogenization of the insect tissues.

Based on these results, the preservation of specimens of the genus *Atta* destined to studies with DNA could be done with any of the methods tested for a period of up to seven months. Except for 95% ethanol at RT, all the other examined methods could be used for preservation of *Atta* spp. up to 12 months. We have found, in agreement with results observed for other insects, that successful DNA extraction from *Atta* spp. can be achieved using a range of preservation techniques, and that there are alternatives to preservation at low temperatures (-70ºC and -20ºC) for storage periods of up to one year, which eliminates the need of high cost freezers.

The effects of each preservation method on DNA quality (considering DNA size above 9.4 kb as indicator of good quality for RAPD analysis), in each storage period are shown in Table 2. The ANOVA indicates a significant effect of the interaction between preservation methods and storage period ($F = 10.67$; $P < 0.05$). All preservation methods produced the same amount of good quality DNA after 90 and 210 days of storage when compared with the control (fresh specimens), although DNA shearing was higher in specimens stored in silica gel and buffer at RT.

Despite satisfactory amounts of good quality DNA, compared with samples stored for 90 days, there was an increase in DNA degradation in all preservation methods after 210 days. After 360 days, DNA extracted from insects preserved in all methods exhibited progressive DNA shearing. Samples preserved at -70ºC, in 95% ethanol at -20ºC and in 95% ethanol at 4ºC did not differ from the control, while insects preserved in silica gel and in buffer at RT showed a significant decrease in DNA quality as compared with fresh specimens. DNA stored in 95% ethanol at RT showed unacceptable results compared with the other methods, lacking the DNA fraction above 9.4. After 360 days of storage, a progressive DNA degradation was observed in all preservation methods when compared with DNA extracted from fresh ants.

The reproducibility of the RAPD banding pattern from samples preserved in the different methods stored at different periods before DNA isolation is shown in Fig. 1 and 2.

Table 2. Quality of DNA (% DNA above 9.4 kb) isolated from *A. sexdens rubropilosa* after storage under different conditions for several periods.

	Storage periods (days)		
Treatments	90	210	360
-70ºC	0.82 a[1] (68.10)[2]	0.69 a (48.68)	0.57 ab (32.50)
Ethanol 95% at -20ºC	0.81 a (66.20)	0.62 a (39.23)	0.55 ab (30.68)
Ethanol 95% at 4ºC	0.78 a (60.85)	0.64 a (41.18)	0.56 ab (32.08)
Ethanol 95% at RT	0.77 a (59.13)	0.71 a (51.15)	0.00 c (0.00)
Silica gel at RT	0.71 a (49.98)	0.58 a (33.98)	0.47 b (23.05)
Buffer at RT	0.73 a (53.53)	0.62 a (38.70)	0.50 b (25.88)
Fresh specimen	0.81 a (65.05)	0.69 a (48.53)	0.70 a (50.00)

[1]Data transformed in arcsine $\sqrt{x}/100$. Means followed by the same letter in a column are not significantly different by Tukey's Test at 5% level of significance.
[2]Original data.

DNA amplification using primer OPB-07 obtained in all preservation methods gave the same banding profile up to 210 days (Fig. 1), indicating that all the treatments maintained the DNA integrity for RAPD analysis. However, different banding pattern was observed with DNA extracted from specimens stored for 360 days in 95% ethanol at RT when compared with other preservation methods. Fig. 2 displays the amplification result using primer OPB-01 and DNA template extracted after 360 days from insects preserved in all methods. The absence of four DNA fragments, approximately 350, 430, 780 and 2200 bp (lanes 7 and 8), demonstrated that DNA obtained after storage in 95% ethanol at RT is not suitable for RAPD analysis. DNA degradation in specimens preserved in alcohol may be due to continuing activity of nucleases, which could be inhibited with the addition of EDTA (Dessauer *et al*. 1990).

Our results clearly indicate that specimens of *Atta* spp. maintained in 95% ethanol at RT for periods longer than 210 days are poor sources of quality DNA for certain molecular techniques such as RAPD, thus leading to unreliable results.

In conclusion, the present study has identified several storage methods that can be used for storing *Atta* spp. specimens for a period of up to seven months, aiming the isolation of high quality DNA for RAPD analysis. For a

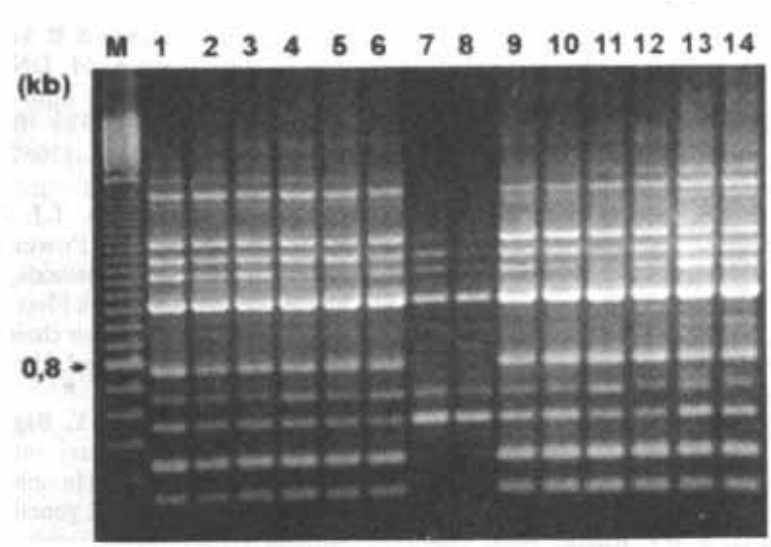

Figure 1. Reproducibility of RAPD profiles with DNA of *A. sexdens rubropilosa* extracted at 210 days (Primer OPB-07). (M) Ladder 100 Marker; Lanes (1-2) -70ºC; (3-4) 95% ethanol at -20ºC; (5-6) 95% ethanol at 4ºC; (7-8) 95% ethanol at RT; (9-10) silica gel at RT; (11-12) buffer at RT; (13-14) fresh specimens.

period of up to 12 months, except for 95% ethanol at RT, *Atta* spp. can be well preserved in any of the other five methods, preferentially in this order: -70ºC, 95% ethanol at -20ºC, 95% ethanol at 4ºC, silica gel and buffer at RT. When the use of low temperatures preservation

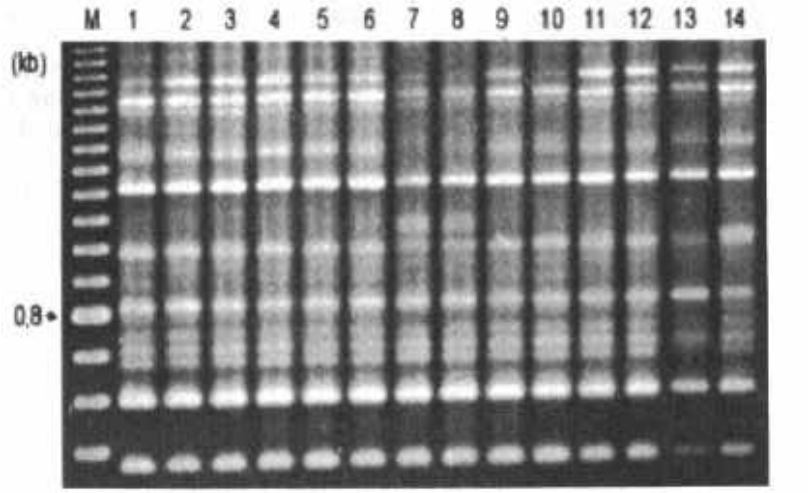

Figure 2. Reproducibility of RAPD profiles with DNA of *A. sexdens rubropilosa* extracted at 360 days (Primer OPB-01). (M) Ladder 100 Marker; Lanes (1-2) -70ºC; (3-4) 95% ethanol at -20ºC; (5-6) 95% ethanol at 4ºC; (7-8) 95% ethanol at RT; (9-10) silica gel at RT; (11-12) buffer at RT; (13-14) fresh specimens.

methods is not possible or the transportation of the material in ethanol is not allowed due to its inflammability, silica gel or buffer solution can be efficient alternatives. These methods are particularly useful for collecting samples under natural conditions, when hazardous substances or special containers are a hindrance.

Literature Cited

Arctander, P. 1988. Comparative studies of avian DNA by restriction fragment lwngth polymorphism analysis: convenient procedures based on blood samples from live birds. J. Ornithol. 129: 205-216.

Bramwell, N.H. & B.F. Burns. 1988. The effects of fixative type and fixation time on the quantity and quality of extractable DNA for hybridisation studies on lymphoid tissue. Expl. Hematol. 16: 730-732.

Cheung, W.Y., N. Hubert, & B. S. Landry. 1993. A simple and rapid DNA microextraction for plant, animal, and insect suitable for RAPD and other PCR analyses. PCR Meth. Appl. 3: 69-70.

Dessauer, H.C., C.J. Cole & M.S. Hafner. 1990. Collection and storage of tissues. p. 25-41. In Hillis, D. M. & C. Moritz (eds). Molecular Systematics, Sinauer, Massachusetts.

Dillon, N., A.D. Austin & E. Bartowsky. 1996. Camparison of preservation techniques for DNA extraction from hymenopterous insects. Insect Mol. Biol. 5: 21-24.

Goelz, S.E., S.R. Hamilton & B. **Vogelstein. 1985**. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem. Biophys. Res. Commun. 130: 118-126.

Koch, D.A., G.A. Duncan, T.J. Parsons, K.P. Pruess & T.O. Powers. 1998. Effects of preservation methods, parasites and gut contents of Black Flies (Diptera; Simuliidae) on polymerase chain reaction products. J. Med. Entomol. 35 : 314-318.

Laulier, M., E. Pradier, Y. Bigot & G. Périquet. 1995. An easy method for preserving nucleic acids in field samples for later molecular and genetic studies without refrigerating. J. Evolution. Biol. 8: 657-663.

Post, R.J., P.K. Flook & A.L. Millest. 1993. Methods for the preservation of insects for DNA studies. Biochem. Syst. Ecol. 21: 85-93.

Reiss, R.A., D.P. Schwert & A.C. Asworth. 1995. Field preservation of Coleoptera for molecular genetic analyses. Environ. Entomol. 24 : 716-719.

Smith, L.J., R.C. Braylan, J.E. Nutkis, K.B. Edmundson, R.R. Downing & E.K. Wakeland. 1987. Extraction of cellular DNA form human cells and tissues fixed in ethanol. Analyt. Biochem. 160: 135-138.

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