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Comparative study on the effect of preservatives and extraction methods on the quality of spider DNA

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A B S T R A C T

Owing to recent innovation in molecular biological techniques, nucleic acid data are becoming more and more important in ecology, systematics, evolutionary biology and conservation biology. Moreover, DNA sequence comparison is a powerful tool to study genetic relatedness of species, which can be used to estimate branching order of phylogenetic trees as well as evolutionary distance between both extinct and extant taxa. To prepare an accurate phylogenetic tree it is necessary to use a preservative which causes less degradation of DNA. It is possible to obtain DNA from dried insects, but this is not an option for arachnids, which require wet preservation to facilitate identification using structures that shrivel when dried. In this context, the effect of three preservatives (acetone, ethanol (70%) and formalin (4%)) on DNA of the spider *Pisaura mirabilis* were tested and compared with fresh specimens in this study. For all treatments, spiders were placed directly in 1.5 ml preservative, kept in room temperature for 3 months and DNA was extracted from the leg of the preserved spider by four different DNA extraction methods (DNeasy method, Quick Extraction method, Master Pure method and CTAB method). PCR quality of the extracted DNA was tested with agarose gel electrophoresis. This study revealed that the quality of the extracted DNA varies and the variation seems related to the mode of preservation and extraction method. From the results it is evident that formalin, which is a routine preservative, has poor capacity to retain the integrity of DNA. Further, ethanol preservation degrades DNA to some extent but gives separation of the strands in extraction. Acetone did not affect the quality of DNA and the yield was comparable to that of fresh specimen. DNA shearing and inter-strand cross linking which occurred both in formalin and ethanol preserved specimens did not occur in acetone. By comparing the results, it can be concluded that acetone is superior to ethanol and formalin in preservation because it yields high quality DNA which can be compared to fresh samples in all the extraction methods tested. Formalin reacts with DNA through interaction with hydrogen bonds, fixation and denaturation of DNA proteins, cross linking between proteins and DNA and methylation of the nucleic acid. Acetone was found to be robust against water contamination than ethanol. Considering that most biological materials contain high amounts of water, acetone may be more recommended as preservative than ethanol which is widely used for the purpose.

Introduction

A powerful approach of studying genetic relatedness of species involves DNA sequence comparisons which can be used to estimate branching order of phylogenetic trees as well as evolutionary distance between extinct taxa (Fleckenstein, 1988). Recent application of the polymerase chain reaction (PCR) and direct sequencing has accelerated efforts to examine a wide range of taxa with DNA comparisons (Kocheret al.1989; Martin et al. 1990). To prepare an accurate phylogenetic tree it is necessary to use a preservative which causes less degradation of DNA.

Most of the studies investigating the effect of preservation techniques on arthropod DNA extraction and quality have been on insects (Post et al.1993; Riess et al. 1995; Dillon et al. 1996; Austin & Dillon, 1997; Quike et al. 1999; Rubink et al. 2003). It is possible to obtain DNA from dried specimens (Post et al. 1993; Dillon et al. 1996) but this is not an option for arachnids, some insects and many other arthropods which are typically stored in ethanol to facilitate identification using structures that shrivel when dried (e.g. the epigyne and the pedipalpal bulb of spiders). Ethanol can affect DNA preservation because of the oxidative and hydrolytic effects. The effects of ethanol and other preservatives have been tested to some extend on insects (Post et al. 1993; Reiss et al. 1995; Dillon et al. 1996). There have been few studies on effects of preservatives on non-insect arthropods (A'Hara et al.1998; Gurdebeke&Maelfait 2002).

Various claims have been made as to the minimum requirements necessary for the preservation of arachnid tissue that will yield useable DNA, but empirical studies on the effect of different DNA preservatives have been limited. A'Haraet al.(1998) found that spider DNA degrade substantially when stored in either ethylene - glycol or 70% ethanol at room temperature. Gurdebeke & Maelfait(2002) tested the effects of three different preservatives (70% ethanol, 4% formaldehyde, and modified Carnoy's solution (acetic acid + TE buffer) and that it was possible to generate a RAPD profile from specimens preserved in 70% ethanol but not from specimens preserved in 4% formaldehyde. Formalin reacts with DNA through interaction with the hydrogen bonds, fixation and denaturation of DNA proteins, cross linking between proteins and DNA and methylation of the nucleic acid (Hamzhaki et al. 1993; Koshiba et al. 1993; Karlsen et al. 1994). Carnoy's solution was also found to be an unsuitable tissue preservative for insect DNA (Post et al. 1993; Riess et al. 1995).

Spiders and other arachnids to be used in DNA studies are typically stored in cold (-20^0C to -80^0C) in 95% - 100% ethanol (Hedin, 1997; Wheeler & Hayashi 1998; Vink et al. 2002). DNA degradation has been reported in spiders stored in 70% - 75% ethanol (Hormiga et al. 2003; Vink& Paterson 2003). A'Hara et al.(1998) found substantial degradation of spider DNA that has been stored in 70% ethanol at room temperature for less than a month. Short segments (<300bp) of mitochondrial DNA (mtDNA) have been amplified from Wolf spiders (Lyosidae) stored in 70% ethanol for up to two years (Colgan et al.2002). However longer segments of mtDNA (>1000bp) are now commonly used in DNA based phylogenetic studies (Hedin &Maddison 2001), requiring DNA that has not been degraded by poor storage methods. When water containing series of acetone and ethanol were examined for DNA preservability, acetone was apparently more robust to water contamination than ethanol (Dessauer et al. 1996).

As described above, previous reports have consistently documented that storage in 70% ethanol is generally recommended for archival preservation of DNA of biological materials. In this context, this study aims to test the effect of the common preservatives (acetone, 70% ethanol and formalin) on the arachnid *Pisaura mirabilis*, a funnel web builder belonging to the family Pisauridae.

Materials and Methods

The arachnid species *Pisaura mirabilis* (Araneae: Pisauridae) was used for the study. The effect of three preservatives (formalin (4%), ethanol (70%), acetone) were tested and compared with fresh specimens. For all treatments, spiders were placed directly in 1.5 ml preservative (as per Prendini et al. 2002). These were kept in room temperature for 3 months and were taken for DNA extraction.

DNA was extracted from the leg of the preserved spider (usually left legs 3 & 4). Muscle tissue in arthropod legs is good source of mitocondrialDNA (mtDNA) (Prendini et al.2002). In the present study a comparison of the effect of the three preservatives (formalin, ethanol and acetone) and the efficiency of the commonly used DNA extraction method is also tested.

Four different methods viz, DNeasy method, Quick Extraction method (QE), Master Pure method (MP) and CTAB method were used for extraction of DNA. The quality of extracted DNA was checked by agarose gel electrophoresis. Fresh sample is used to compare the effect of preservative in each extraction method.

Results and Discussion

The quality of the extracted DNA, by four different extraction methods viz, DNeasy, QE, MP, CTAB which were preserved in 3 different preservatives vary and the variation seems related to the mode of preservation and extraction method.

Plate-1 show the DNA strands on the gel plate extracted by the four different methods, preserved in 3 different preservatives. The plate is stained with EtBr and viewed in UV light. There are 17 lanes of which the lane 0 is the DNA ladder strands ranging from 500bp-2000bp, which is used to compare the concentration and size of the strands obtained from the samples.

Lanes 1-3 represents the DNA preserved in formalin, ethanol and acetone and lane 4 is the control sample. All these are extracted by DNeasy method. Lanes 5 - 7, DNA extracted using Quick extract method which preserved in formalin, ethanol and acetone respectively. Lane 8 is the control for this extraction method. Lanes 9-11 DNA extracted by Master pure method, and the control for this extraction on lane 12. Lanes 13 - 16 extracted using CTAB method. DNA preserved in different preservatives on lane 13 - 15 and on lane 16 control of this method. Agarose gel electrophoresis show that the bands obtained after extraction by all the four methods are <2000bp.

Effect of formalin and extraction methods on DNA

In Plate1 lanes 1, 5, 9 and13 represents DNA strands preserved in 4% formalin. The specimens preserved in formalin yielded little or no banding pattern when extracted. Thus formalin proves to be a poor preservative for molecular analysis.

Extraction methods have also influenced DNA. It is shown that all three extraction methods namely (MP, QE, DNeasy) did not

yield any DNA strands with formalin preserved DNA, but a little or feeble band formation is obtained when CTAB method is used for extraction. DNA is highly degraded when formalin is used as a preservative.

Effect of ethanol and extraction methods on DNA

In Plate1 ethanol preserved DNA lanes are 2, 6, 10 and 14. The yield of DNA is less compared to acetone and control specimen. The separation of DNA is not evident or band is formed only to some extent. When compared to formalin it gives better results. Ethanol is used as a routine preservative but the results of the extraction methods show that there is degradation of DNA.

Even though the bands obtained by ethanol are degraded, it shows the maximum separation in CTAB method than other 3 methods (MP, QE and DNeasy). Degradability of ethanol is less compared to formalin.

Effect of acetone and extraction methods on DNA

It is clear that acetone gives better results when compared to all the other preservatives. The banding pattern observed is similar to the control samples. So there is only little degradation when acetone is used.

Extraction method also shows positive results. In every extraction method high quality banding pattern was observed and marked band formation was observed in CTAB method. Thus acetone can be recommended as a preservative for molecular studies.

Earlier records show that the morphological features was the only criteria for classification. Following the proposal of the structure, physical and chemical properties of DNA in the earlier half of twentieth century, marked the beginning of molecular taxonomy. Morphological features are a result of the particular proteins produced by specific genes; classification will be accurate only if molecular taxonomy is used. For this the nuclear material should maintain its integrity, but this depends on the quality of preservation techniques used. Hence it is necessary to preserve DNA in a preservative with minimal degradation.

Degradation of DNA occurs when endo and exo nuclear activity cleaves the DNA strand and breaks it up into small fragments which are no longer suitable for further molecular analysis (Linn 1981). Ester linkages with phosphate molecule and carbon-nitrogen linkages are especially susceptible to modification in DNA (Cann et al. 1993).

Effect of Formalin on DNA

From the results it is evident that formalin, which is a routine preservative, has poor capacity to retain the integrity of the DNA. This result is concordant with the previous reports that formalin preserved sample yielded no visible DNA after EtBr staining. (Mething et al. 2006; Postet al.1993; Gurdebeke&Maelfait2002). This is because formalin reacts with DNA through interaction with the hydrogen bonds, fixation and denaturation of DNA proteins, crosslinking between proteins and DNA and methylation of nucleic acid (Hamazaki et al. 1993; Koshibaet al. 1993; Karlsen et al. 1994).

The unsuccessful extraction of DNA from the formalin fixed specimens that were preserved for 3-4 years indicate the effect of aldehyde fixatives in causing significant degradation of DNA over a long period of

time (Chakraborthy et al. 2005). It is an established fact that formalin derivatives interfere with many molecular techniques by forming complexes or crosslinks and hindering PCR amplification of marker sequences. Koshiba et al. (1993) reported that extensive DNA degradation occurs during formalin fixation because of the presence of formic acid coupled with low P^H and low salt concentration at room temperature. These results are complementary to the results obtained in this study at room temperature.

Formalin is used as a routine preservative in museums and these specimens are used by researchers for phylogenetic and taxonomic studies. From the results of this study it is evident that 3 months preservation in formalin degrades the DNA. Vochot&Monnerot(1996) have observed considerable DNA degradation in samples fixed for 24 hrs at room temp and this suggest that specimens stored in museums for several years will not give correct information regarding the phylogenetic and taxonomic position of the specimen.

Another discrepancy regarding this is that Chakraborthy et al. (2005) suggested that specimens fixed for a short duration (7days) is not enough to bring about changes or degradation which is in controversy to the findings of Vachot&Monnerot(1996) who observed degradation in sample fixed in formalin for 24 hrs at room temperature.

It has been suggested that formalin fixation time and temperature at which sample is fixed are critical factors for degradation of endogenous DNA (Vachot&Mannerot1996). Koshiba et al. (1993) suggested that the use of buffered formalin at room temperature permitted extraction of DNA of sufficient quantity. Chakraborthy et al. (2005) suggested that the use of formalin at 4°C also gave good results. The discrepancies above suggest the need for further investigation to analyse the effects of storage conditions on DNA preservation.

Effect of Ethanol on DNA

Results of this experiment indicate that ethanol preservation degrades the DNA to some extend but it gives separation of the strands in extraction. Substantial degradation of spider DNA was found after storage for 3 weeks in 70% ethanol at room temperature (A' HARAet al. 1998). This degradation was also reported by Seutin et al. (1991); Holzmann&Pawlowshi(1996) and Zhang & Hewitt (1998).

The different extraction methods used also show difference in banding pattern. The same was observed by Chakraborthy et al. (2005). Ethanol preserved specimens appeared to give more accurate results than formalin, although there were difference in the success rates among various extraction methods. Ethanol preserves the specimen by the inhibition of cellular enzymes such as DNAses in an irreversible manner and hence the quality of DNA remains intact or undergoes negligible degradation over a period of time (Chakaraborthy et al. 2005). Shiozawa et al. (1992) reported that yields of DNA obtained from ethanol preservation were equal to those from frozen tissue and superior to formalin preservation.

According to Shedlock et al. (1997), ethanol preserved specimens gave better yields of DNA than do formalin because the DNA remains associated with protein complex and degradation will be lesser than that of formalin. In contrast to the result obtained in this study Vink et al. (2005) reported that specimens preserved in 70% ethanol at room temperature did not yield DNA but when stored at -20°C and -80°C

gave better results. -80°c is reported to be the appropriate temperature for ethanol preservation (Post et al. 1993; Riess et al. 1995; Dillon et al. 1996).Vink et al. (2005)found that 100% ethanol preserved Hymenoptera, could be effectively used for upto 16 months. DNA degradation occurs in tissue stored at room temperature in 95% ethanol for six weeks.

These discrepancies from the present result may be due to the concentration difference and also the temperature at which it was stored. Chakraborthy et al. (2005) reported that the concentration and temperature play vital role in preservation of DNA. Arctander (1985) preserved specimens in 70% ethanol at room temperature by mincing the specimen in the preservative and immediately replacing the spoiled alcohol with fresh alcohol.A significant increase in both DNA quality and extraction yield was obtained by moth preservation in 100% ethanol at 4°C, whereas preservation in 75% ethanol gave poor results in both room temperature and at 4°C, suggesting that only 100% ethanol offers reasonable DNA preservation.

Effect of Acetone on DNA

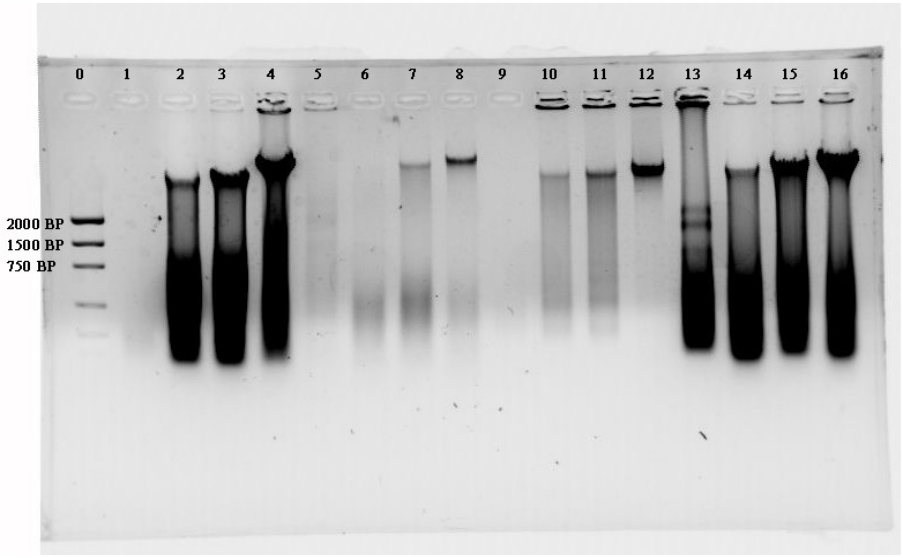
Acetone preservation did not affectthe quality of DNA and the extraction yield was comparable to that of fresh specimen. DNA shearing and interstrand cross linking which occurred both in formalin and ethanol preserved specimens did not occur in acetone.

It was demonstrated that 100% acetone preserved the DNA after 6 months storage as efficiently as 100% alcohol (Mandrioli et al. (2006). Flournoy et al. (1996) reported robustness of acetone in H_2O contaminated specimens. Since all biological materials contain H_2O, its robustness is the merit of acetone method to ethanol method. Acetone preserves the DNA of biological materials better than ethanol in the presence of H_2O. However it is conceivable that the high penetrability and dehydrating activity of acetone may be responsible (Mandriol et al. 2006). Mandrioli et al. (2006) also reported that various tissues could be stored in acetone without any degradation in a condition suitable for molecular analysis for several years.

In biological materials, DNA is easily degraded by intrinsic enzymes such as nucleases and peroxidases. Even in the absence of such enzymatic activity DNA spontaneously decays due to hydrolyses oxidation and non-enzymatic methylation (Lindahl, 1993). Acetone quickly denatures and inactivates enzymes in tissues and cells. Dehydration will suppress the progress of hydrolytic decomposition of DNA. These properties make acetone a better preservative than formalin and ethanol (Fukatsu, 1999). It was demonstrated that in addition to DNA, proteins were also preserved in acetone. Many proteins were extracted from insects and could be used for molecular analysis after 2 years of storage in acetone (Fukatsu, 1999).

Acetone can be substituted for standard histological fixatives such as formalin, picric acid and mercuric chloride. Notably acetone has a great advantage over these fixatives as it is difficult to extract DNA from materials in standard histological fixatives (Goelz et al. 1985). Acetone preservation proves to be cost effective and widely applicable compared to ultra cold freezers and liquid nitrogen making it excellent storage medium not only during field surveys but also to obtain efficient and low cost storage of museum specimens.

Plate.1 Autoradiogram showing the result of different DNA extraction methods and preservatives



References

A'Hara, S., Harling, R., McKinlay, R.G.&Topping, C. J. (1998).RAPD profiling of spider (Araneae) DNA. Journal of Arachnology 26, 397-400.

Arctander, P. (1988). Comparative studies of avian DNA by restriction fragment length polymorphism analysis: convenient procedures based on blood samples from live birds. Journal ofOrnithology, 129, 205–216.

Austin, A. D.&Dillon, N. (1997).Extraction and PCR of DNA from parasitoid wasps that have been chemically dried.Australian 2nd journal of Entomology 36, 241-244.

Cann, R.L., Feldman, R. A., Freed, L.A., Lum, J. K. &. Reeb, C. A.(1993). Collection and storage of vertebrate samples. Methods in Enzymology 224:38–51.

Chakraborthy, A., Sakai, M.&Iwastsuki, Y. (2005). Museum fish specimen and molecular taxonomy: A comparitive study on DNA extraction protocols and preservation technique. J. Appl. Ichtyol. 22, 160-166.

Dessauer, H. C., Cole, C. J. &Hafner, M. S. (1996). Collection and storage of tissues. In: (edsHillis DM, Moritz C, Mable BK) MolecularSystematics, 2nd edn, pp. 29–47. Sinauer Associates, Sunderland.

Dillon, N., Austin, A. D.&Batowsky, E. (1996).Comparison of preservation techniques for DNA extraction from hymenopterous insects. Insect Molecular Biology 1933–1936.5, 21-24.

Felsenstein, J. (1988). Phylogeniesnfrom molecular sequences : inference and reliability. Ann. Rev. Gen., 22: 521-565.

Flournoy, L. E., Adams, R. P.&Pandy, R.N. (1996).Interim and archival preservation of plant specimens in alcohols for DNA studies.Biotechniques, 20, 657–660.

Fukatsu,T.&Shimada, M. (1999). Molecular characterization of Rickettsia sp. in a bruchid beetle Kytorhinussharpianus.

AppliedEntomology and Zoology, 34, 391–397.

Goelz, S. E., Hamilton, S. R.&Vogelstein, B. (1985). Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochemical and Biophysical Research Communications, 130, 118–126.

Gurdebeke, S.&Maelfait, J. P. (2002). Pitfall trapping in population genetics studies: finding the right "solution". The Journal of Arachnology 30, 225-261.

Hamazaki, S., Koshiba, M., Habuchi, T., Takahashi, R.&Sugiyama, T. (1993). The effect of formalin fixation on restriction endonuclease digestion of DNA and PCR amplification. Path Res Pract 189: 553–557.

Hedin, M. C.(1997). Molecular phylogenetics at the population /species interface in cave spiders of Southern Applachians (Araneae: Nesticidae: Nesticus). Molecular Biology and Evolution 14, 309-324.

Hedin, M. C. &Maddison, W. P (2001). A combined molecular approach to phylogeny of the jumping spider subfamily Dendryphantinae (Araneae: Salticidae).Molecular Phylogenetics and Evolution 18, 386-403. doi:10. 1006/mpev. 2000.0883.

Holzmann, M. & J. Pawlowski.(1996). Preservation of Foraminifera for DNA extraction and PCR amplification. Journal of Foraminiferal Research 26:264–267.

Hormiga.G., Arnedo, M. A.&Gillespie, R. G.(2003). Speciation on a conveyor belt : sequential colonization of the Hawaiian Islands by Orsonwelles spiders (Araneae, Linyphiidae). Systematic Biology 52, 70-88.

Karlsen, E., Kalantari, M.,Chitemerere, M., Johannson, B. &Hagmar, B. (1994).Modifications of human and viral deoxyribonucleic acid by formaldehyde fixation. Lab Invest 71:604–611.

Kocher, T. D., Thomas, W. K., Meyer, A., Edward, S. V., Paabo, S., Villablanca, F. X. &Wilson, A .C (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and Sequencing with conserved primers. Proc. Natl. Acad. Sci. USA, 86:6196-6200.

Koshiba, M., Ogawa, K., Hamazaki, S.; Sugiyama, T., Ogawa, O.&Kitajima, T. (1993). The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded Tissues. Pathol. Res. Pract. 189, 66–72.

Lindahl, T. (1993).Instability and decay of the primary structure of DNA.Nature, 362, 709–715.

Mandrioli, M., Borsatti, F.&Mola, L (2006).Factors affecting DNA preservation from museum-collected Lepedopteran specimens.EntamologiaExperimental is et Applicata 120: 239-244.

Martin, A.P., Kessing,B. D. &Palumbi, S. R. (1990). The accuracy of estimating genetic distance between species from short sequences of mitochondrial DNA.Mol. Biol. Evol., 7:485-488.

Meithing, F., Hering, S., Hansche, B.&Pressler, J (2006).Effect of fixation to degradation of nuclear and mitochondrial DNA in different tissues. Journal of Hiistochemistry and Cytochemistry 54(3): 371-374.

Post, R. J., Flook, P. K.&Millest, A. L. (1993). Methods for the preservation of insects for DNA

studies.Biochemical Systematics and Ecology**21**, 85–92.

Prendini, L., Hanner R. &DeSalle, R.2002. Obtaining, storing and archiving specimens and tissue samples for use in molecular studies. 1-78.

Quicke, D. L. J., Beishaw, R.&Lopez-vaamode, C. (1999).Preservation of hymenopteran specimens for subsequent molecular and morphological study.Zoologiascripta 28, 261-267. doi:10. 1046/ j.1463-6409.1999.00004.x.

Riess, R.A., Schwert, D. P.&Asworth, A.C. (1995).Field preservation of coleopteran for molecular genetics analyses. Molecular Entomology 24,716-719.

Rubink, W. L., Murray, K. D., Baum, B. A.&Pinto, M. A. (2003). Long term preservation of DNA from honey bees (Apismellifera) collected in ariel pitfall traps. The Texas Journal of Science 55, 159-168.

Seutin, G., White, B. M. &Boag, P. T. (1991). Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology 69:82–90.

Shedlock, A. M.; Haygood, M. G.; Pietsch, T. W.&Bentzen, P. (1997).Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens.BioTechniques 22, 394–400.

Shiozawa, D. K., Kudo, J., Evans, R. P., Woodward, S. R.&Williams, R. N. (1992). DNA extraction from preserved trout tissues. Great Basin Nat. 52, 29–34.

Vachot, A. M.&Monnerot, M. (1996). Extraction, amplification and sequencing of DNA from formaldehyde-fixed specimens. Ancient Biomol.1:3.

Vink, C. J.&Paterson, A. M. (2003). Combined molecular and morphological phylogenetic analyses of the New Zealand wolf spider genus Aneteropsis (Araneae: Lycosidae). Molecular Phylogenetics and Evolution 28, 576-581.doi:10.1016/S 1055-7903(03) 00219-7.

Vink, C. J., Mitchell, A. D.&Paterson, A. M. (2002).A preliminary molecular analysis of phylogenetic relationships of Australian wolf spider genera (Araneae: Lycosidae). The Journal of Arachnology 30, 227-237.

Vink, C.J., Thomas, M. S., Paquin, P., Hayash, C. Y.&Hedin, M. (2005).The effects of preservatives and temperatures on arachnid DNA. Invertebrate Systematics 19 , 99-104.

Wheeler, W. C.&Hayashi, C. Y. (1998).The phylogeny of the extant chelicerateordrs. Cladistics 14, 173-192. doi:10. 1006/clad. 1998.0061.

Zhang, D. X. & Hewitt, G. M. (1998). Field collection: animals. Pp. 46–48. In Molecular Tools for Screening Biodiversity. (A. Karp, P.G. Isaac & D.S. Ingram, eds.). Chapman & Hall, London.