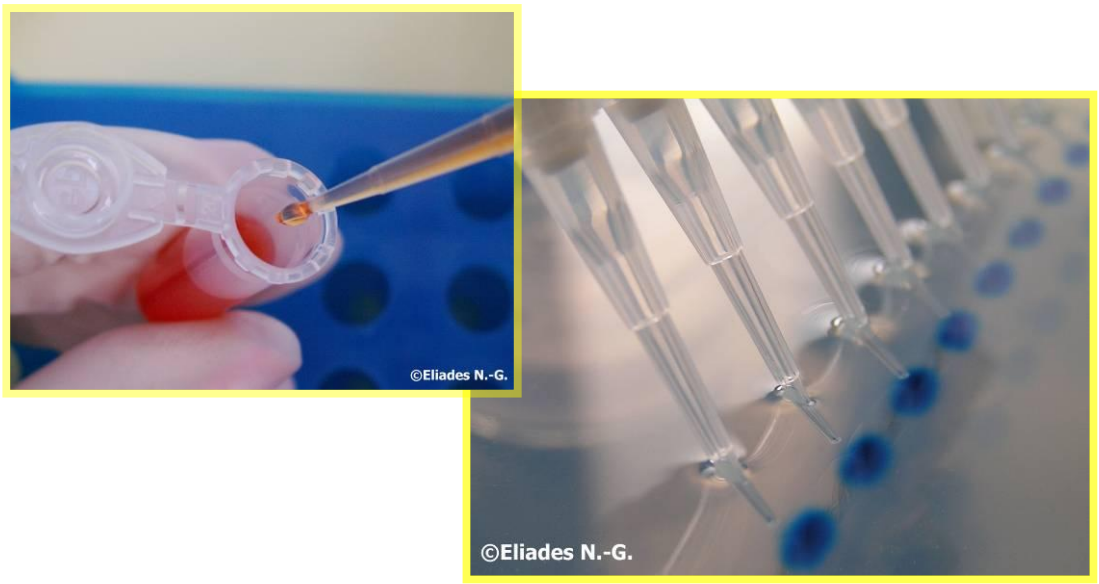
FREDERICK RESEARCH CENTRE

Vulnerability of the narrow endemic *Cedrus brevifolia* from Cyprus: Detection of genes and phenotypic trait diversity linked to adaptation.

Protocol No. DIDAKTOR/0609/13

Deliverable 13



Principles and Laboratory Protocols of Molecular Techniques Employed in assessments for the present project.

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[1] The present report has been prepared by Ecologie des forêts Mediterraneennes, INRA, Avignon in the framework of the project DIDAKTOR/0609/13 entitled "Vulnerability of the narrow endemic *Cedrus brevifolia* from Cyprus: Detection of genes and phenotypic trait diversity linked to adaptation."The project is co-financed by the European Regional Development Fund and the Republic of Cyprus through the Research Promotion Foundation.

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1. Introduction

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics (Holloway et al. 2007). In the last three decades the molecular and biochemical markers have been of crucial importance in forest genetics investigating genetic variation with regard to its spatial and temporal heterogeneity within and among populations (Weising et al. 2005). The genetic markers could be defined as: (i) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA or (ii) a specific piece of DNA with a known position on the genome or (iii) a gene whose phenotypic expression is usually easily discerned, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, or chromosomes, or locus (see Semagn et al. 2006).

The development of polymerase chain reaction (PCR) method, which is a molecular biology technique for enzymatically replicating (amplifying) small quantities of DNA without using a living organism, could be regarded as the major technique for the increased molecular markers application in genetics. It is used to amplify a short (usually up to 10 kb), well-defined part of a DNA strand from a single gene or just a part of a gene. According to the primers used for amplification (in molecular markers), PCR-based techniques can be classified in two types: (i) Arbitrary or semi-arbitrary primed PCR techniques that have developed without prior sequence information (e.g. *AP-PCR, DAF, RAPD, AFLP, ISSR*) and (ii) Site-targeted PCR techniques that have developed from known DNA sequences (e.g. *EST, CAPS, SSR, SCAR, STS*).

The scientific objective of this project is to investigate the adaptation and adaptability of *Cedrus brevifolia* (Cyprus cedar) through the correlation of various datasets: genes diversity, morphological / anatomic traits and ecological indexes (soil conditions and climatic measurements). Hence, outcomes from this project aim to shed light on the adaptation process of Cyprus cedar in the course of many generations due to the interaction of several evolutionary processes (mostly selection owing to glaciations and demographic factors).

Detailed studies of individual genes have shown that gene expression divergence often results from adaptive evolution of a regulatory sequence. Genome-wide analyses, however, have yet to unite patterns of gene expression with polymorphism and divergence to infer population genetic mechanisms underlying expression evolution (Holloway et al. 2007). The same authors (Holloway et al. 2007) argued that the changes in gene expression are governed primarily by the evolution of *cis*-acting and *trans*-acting factors.

The assessment of *C. brevifolia* gene adaptation and gene adaptability will be carried out by direct investigation of genetic structure in the candidate genes (see Deliverable 9, Table 1, Table 2, Table 3). The research strategy for the molecular labwork is separated in two sections: (i) testing the transferability and successful amplification of numerous genes to a low number of *C. brevifolia* samples and (ii) applying successfully amplified genes to all samples (see Deliverable 11, Table 2), after careful and complete pre-evaluation of results (genes successful amplification and gene diversity) of adaptive candidate genes from the previous step (i).

The gene diversity of candidate genes assess directly on genes sequences, and, hence, single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) will be detected. Several studies argued that SNPs and InDels are highly abundant and distributed throughout the genome in various species including plants (i.e. Garg et al. 1999; Batley et al. 2003). In general, SNPs genotyping assays to one of four groups based on the molecular mechanism: (i) allele specific hybridization, (ii) primer extension, (iii) oligonucleotide ligation and (iv) invasive cleavage

(Sobrino et al. 2005). In all the cases SNP genotyping combines two elements: (i) the generation of an allele-specific product and (ii) the analysis thereof. In the present study the methods of primer extension are used. Primer extension is based on the ability of DNA polymerase to incorporate specific deoxyribonucleotides complementary to the sequence of the template DNA. There are several variations of the primer extension reaction, which can be divided into three main types of reaction.

This deliverable (Deliverable 13) aims to present the principles and laboratory protocols of molecular techniques employed until now in this project. However, it should be mentioned that these protocols could be optimised through future labwork and should therefore not be assumed to be the final protocols.

2. Protocols applied in the investigation of genetic structure in *Cedrus brevifolia*

2.1 DNA isolation

DNA isolation was performed using the protocol Qiagen DNeasy96™ Plant kit (*Qiagen*). The DNA has been isolated from all specimens by using fresh needles. The amount and quality of the DNA was analysed by 0.8% agarose gel electrophoresis with 1X TAE as running buffer (Sambrook et al. 1989). DNA was stained with ethidium-bromide and visualized by UV illumination. The DNA quality was found to be good and, hence, genetic analysis began immediately.

The DNeasy 96 Plant Kit (Qiagen 2004) contains:

DNeasy 96 Plant Kit

DNeasy 96 Plant Kit (6)	
Catalog no.	**69181**
Number of preps	**6 x 96**
DNeasy 96 Plates	6
S-Blocks*	2
Collection Microtubes, 1.2 ml (racked)	12 x 96
Collection Microtube Caps	4 x (120 x 8)
Elution Microtubes RS (racked) and caps	6 x 96
AirPore Tape Sheets	5 + 25
Buffer AP1	2 x 140 ml
Buffer AP2	90 ml
Buffer AP3/E (concentrate)†‡	125 ml
Buffer AW (concentrate)‡	2 x 81 ml
Buffer AE	128 ml
RNase A (100 mg/ml)	2 x 440 µl
Reagent DX	1 ml
96-Well-Plate Registers	6
Handbook	1

* Reusable; see Appendix B (page 50) for cleaning instructions.

† Contains a chaotropic salt. Not compatible with disinfectants containing bleach. See page 7 for safety information.

‡ Buffer AP3/E and Buffer AW are supplied as concentrates. Add ethanol (96–100%) according to the bottle label before use to obtain a working solution.

Preparation activities before starting

- Buffer AW and Buffer AP3/Ewere supplied as concentrates. Before using for the first time, the appropriate amount of ethanol (96 – 100%) was added as indicated on the bottle, to obtain a working solution.
- Buffer AP3/E concentrate may form precipitates upon storage. When necessary, the buffer was warmed to 65 °C to redissolve (before adding ethanol). Buffer AP3/E was not heated after ethanol has been added.
- Buffer AP1 was preheated to 65 °C. This heating was necessary for the DNeasy 96 Plant procedure, and also dissolved any precipitate that may have formed in Buffer AP1.

Method/Procedure

1. Plant tissues were collected and placed up to 50 mg (max) into each tube in 2 collection microtube racks.
2. One tungsten carbide bead was added to each collection microtube.
3. Buffer AP1, RNase A, and Reagent DX were combined, according to the table below, to make a working lysis solution. 400 µl of the working lysis solution was pipeted into each collection microtube. Microtubes were sealed with the caps provided. (It is important to prepare a fresh working lysis solution).

To allow thorough mixing of the solution, the components were combined in a tube and vortexed to mix; then the solution was dispensed into a reagent reservoir for use with a multichannel pipet.

	Volume per sample	Volume for 2 x 96 samples*
Buffer AP1 (preheated to 65°C)	400 µl	90 ml
RNase A (100 mg/ml)	1 µl	225 µl
Reagent DX†	1 µl	225 µl

* 15% excess mixture is included in these calculations to allow for pipetting errors.

† Reagent DX is viscous.

4. Each rack of collection microtubes was sandwiched between adapter plates and fixed into TissueLyser clamps.
   - It was made sure that the microtubes were properly sealed with caps. Two plate sandwiches were clamped to the TissueLyser to provide balance. When processing 96 samples or less, a second plate sandwich was assembled using a rack of collection microtubes containing tungsten carbide beads, but no samples or buffers, and was fixed into the empty clamp.
5. Samples were grinded for 1.5 min at 30 Hz.
   - Prolonging the disruption time was avoided, as this could result in DNA shearing.
6. Plate sandwiches were removed and disassembled. Collection microtubes were ensured to be tightly closed. Plate sandwiches were reassembled so that the collection microtubes nearest the TissueLyser in steps 4 and 5 were now outermost. Plate sandwiches were reinserted into the TissueLyser.

   - Rotating the racks of collection microtubes in this way ensured that all samples were thoroughly disrupted. Merely rotating the entire plate sandwich so that the QIAGEN logos were upside down when reinserted into the mixer mill would not have been sufficient, since the same samples that were outermost during the initial disruption would remain outermost in the second disruption step.

7. Samples were grinded for another 1.5 min at 30 Hz.

    - Prolonging the disruption time was avoided, as this could result in shearing of DNA.

8. Plate sandwiches were removed from the TissueLyser and adapter plates were removed from each rack of collection microtubes. To collect any solution from the caps, collection microtubes were centrifuged. Centrifuged was allowed to reach 3000 rpm, and then stopped.

9. Caps were removed and discarded. The130 µl Buffer AP2 was added to each collection microtube.

10. Microtubes were closed carefully with new caps. A clear cover was placed over each rack of collection microtubes and racks were shaken vigorously up and down for 15 s. To collect any solution from the caps, collection microtubes were centrifuged. Centrifuge was allowed to reach 3000 rpm, and then stopped.

    Note: To ensure optimal DNA yields, racks of collection microtubes were vigorously shaken up and down with both hands for the full 15 sec. The genomic DNA is not sheared by vigorous shaking. The centrifugation step prevents precipitates from freezing to the caps, which would otherwise be difficult to remove after incubation at –20 °C (step 11).

11. Racks of collection microtubes were incubated for 10 min at -20 °C. This incubation aids the precipitation of proteins and inhibitors of downstream applications following addition of Buffer AP2.

12. Racks of collection microtubes were centrifuged for 5 min at 6000 rpm. Compact pellets were formed.

    - Care was taken not to transfer any of these particles in the following step.

13. Caps were removed and discarded. 400 µl of each supernatant was transferred to new racks of collection microtubes, ensuring that the new tubes were in the correct orientation.

    - No more than 400 µl of the supernatant was transferred, as otherwise the capacity of the DNeasy 96 plates and the S-Blocks used in subsequent steps would be exceeded. When less than 400 µl supernatant was recovered, the amount of Buffer AP3/E in step 14 was adjusted accordingly.

14. 1.5 volumes (typically 600 µl) of Buffer AP3/E were added to each sample.

    - It was ensured that ethanol was added to Buffer AP3/E prior to use.

15. Collection microtubes were closed with new caps; ensuring that the tubes were properly sealed to prevent leakage during shaking. A clear cover was placed over each rack of collection microtubes and racks were shaken vigorously up and down for 15 sec. To collect any solution from the caps, collection microtubes were centrifuged. Centrifuge was allowed to reach 3000 rpm, and then stopped.

    - To ensure optimal DNA yields, racks of collection microtubes were vigorously shaken up and down with both hands for the full 15 sec. The genomic DNA is not sheared by vigorous shaking.

16. Two DNeasy 96 plates were placed on top of S-Blocks (provided by the manufacturer). DNeasy 96 plates were marked for later sample identification.

17. Caps were emoved and discarded from the collection microtubes. 1 ml of each sample was carefully transferred to each well of the DNeasy 96 plates. Care was taken not to wet the rims of the wells to avoid aerosols during centrifugation. No more than 1 ml was transferred per well.

    - Lowering pipet tips to the bottoms of the wells could cause sample overflow and cross-contamination. Therefore, one set of caps was removed at a time, and drawing up the samples was begun as soon as the

pipet tips contacted the liquid. This was repeated until all the samples have been transferred to the DNeasy 96 plates.

18. Each DNeasy 96 plate was sealed with an AirPore Tape Sheet and centrifuged for 4 min at 6000 rpm. AirPore Tape prevents cross-contamination between samples during centrifugation. After centrifugation, all of the lysate was checked to have passed through the membrane in each well of the DNeasy 96 plates. When lysate remained in any of the wells, it was centrifuged for a further 4 min.
19. The tape was removed. 800 µl Buffer AW was added to each sample.
20. DNeasy 96 plates were centrifuged for 15 min at 6000 rpm to dry the DNeasy membranes. For efficient drying, the DNeasy 96 plate was not resealed with AirPore Tape.

    - Residual ethanol in the DNeasy membranes derived from Buffer AW may inhibit PCR and was therefore removed by centrifugation before elution of the DNA. DNeasy membranes were sometimes slightly colored after this wash step. This did not affect the DNeasy 96 Plant procedure. A very dark membrane could indicate that too much starting material was used.

21. To elute the DNA, each DNeasy 96 plate was placed in the correct orientation on a new rack of Elution Microtubes RS (provided), 100 µl Buffer AE was added to each sample, and DNeasy 96 plates were sealed with new AirPore Tape Sheets (provided by the manufacturer). The DNeasy 96 plates were incubated for1 min at room temperature (15 – 25°C) and then centrifuged for 2 min at 6000 rpm. Elution in 2 x 50 µl (instead of 2 x 100 µl) increases DNA concentration, but decreases the overall DNA yield.
22. Step 21 was repeated with another 100 µl Buffer AE. New caps were used to seal the Elution Microtubes RS for storage.

Note: This protocol describes processing of 192 samples (2 x 96) and all centrifugation steps were performed at room temperature.

2.2 Electrophoresis Gel

2.2.1 *Agarose gel electrophoresis*

Agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the “stiffer” is the gel. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques.

- *Solutions*

Electrophoresis buffers:

1x TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 (adjust pH with glacial acetic acid)

- Electrophoresis buffers were prepared as 50x (TAE) concentrated stock solutions and diluted prior to use.

Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in electrophoresis buffer or water

Agarose: 0.3 to 2% in electrophoresis buffer

Staining solution: 1 µg/ml ethidium bromide in electrophoresis buffer

Method/Procedure

1. Agarose was suspended at the desired concentration in an appropriate amount of electrophoresis buffer in a bottle or flask (e.g., 1 gr of agarose per 100 ml of electrophoresis buffer yields a 1% gel). The gel slurry did not occupy more than 50% of the bottle. Flasks were covered, and bottles were loosely capped.
2. The suspension was boiled in a microwave oven, until the agarose was completely dissolved. After complete melting, the solution was clear and free of particles.
3. The agarose solution was taken out of the oven and put on a magnetic stirrer to let it cool down, allowing to cool to 60 °C. Stirring the solution prevented uneven cooling. In the meantime, the edges of the plastic tray supplied with the electrophoresis apparatus were sealed using tape. Aslot-forming comb was inserted.

   - Band resolution is, to some extent, dependent on the shape of the teeth of the comb: sharp teeth yield sharp bands, but also allow less volume to be applied. The teeth were checked not to be too close to the bottom of the gel mold. Fine holes in the bottom of a slot might allow the sample to escape in an undesired direction.

4. The gel mold was ensured to be in a horizontal position. The agarose was slowly poured into the gel mold, removing small air bubbles and allowing the agarose to solidify.
5. When the agarose was solid, the gel mold was insertedinto an electrophoresis apparatus filled with TAE buffer.
6. Samples were loaded into the submerged slots (mix of 5µl DNA + 2 µl, 1:4 bromophenol blue).
7. Current was allowed to flow into the system using the power supplier (100 – 200 mA for 20 - 45 min).
8. After the run was completed, the gel was remobed from the apparatus and stained for 20 - 30 minutes with ethidium bromide solution.

   Note: Staining of agarose gels can be achieved in two ways: (1) by adding ethidium bromide to the cooled agarose solution as well as to the running buffer prior to electrophoresis, or (2) by staining the gel after electrophoresis in buffer or water containing ethidium bromide. Ethidium bromide is usually prepared as a stock solution (10 mg/ml) and stored in the refrigerator in the dark. In the present study the first way was followed.

Ethidium bromide-stained gels were documented on a UV transilluminator at 302 nm using either a commercial documentation system equipped with a video camera and a computer (camera system with an orange).

2.2.2 Polyacrylamide gel (PAA)

Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Acrylamide is a potent neurotoxin and was therefore handled with care. Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

- Solution and chemicals

- Acrylamide stock solution: 38% acrylamide and N´,N´-methylene bisacrylamide (19:1 ratio) (Monomers of acrylamide and bisacrylamide are neurotoxic)
- 10x TBE buffer [2.25 mM Tris-borate, 1mM EDTA, pH 8.0 (adjust pH with boric acid)]
- 13-15 µL TEMED
- 125 µL Ammonium Persulfate Solution (10%)
  Note: Freshly prepared; or stored at 4 °C for less than 1 week
- 4-5 ml Loading Ranger Buffer (0.25% bromophenol blue, 60 mM EDTA, 30% glycerol)

Method/Procedure

1. The two thoroughly cleaned glass plates of the electrophoresis apparatus were clamped with spacers in between.
2. 8-10 ml of acrylamide stock solution was mixed with 2.5 ml of 10x TBE and 37 ml H_2O and deaerated under vacuum for 10 min.
3. 25-29 µL of TEMED and 250 µL of 10% ammonium persulfate solution were added. The solution was mixed gently, avoiding air bubbles.
4. The solution was poured between the plates and the slot-former was put in place.
5. Polymerization took approximately 1 h.
6. The glass plates with the gel were inserted into the electrophoresis apparatus. (The tape between the glass plates and the spacer on the bottom was removed).
7. The electrophoresis tanks were filled with lx TBE, the slot-forming comb was removed, and the wells were thoroughly cleaned using a pipet.
8. The DNA sample was mixed with 0.2 vol of loading buffer. Samples were concentrated when volumes were too large: (1) by precipitation of the DNA and dissolving in a smaller volume, and (2) by using a vacuum concentrator. Special narrow tips were used to deposit the sample at the bottom of the well.
9. The gel was run at 500 V as well as a cooling system (20 °C).
10. After electrophoresis (3-4 h), the gel was removed from both glass plates, and stained with silver nitrate.

    - Silver staining solution (0.01% solution, 30 µl into 300 ml bidistillated water) was covered with aluminium folio to avoid light

11. After 30 min the gel was taken out, put on a UV illuminator and finally a picture was taken.

2.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is based on the enzymatic in vitro amplification of DNA. PCR method includes three major steps: (ii) The double-stranded DNA is denatured at high temperature (92 - 95 °C) to form single strands (templates), (ii) Short single strands of DNA (known as primers) bind at a lower annealing temperature to the single stranded complementary templates at ends flanking the target sequences and (iii) the temperature is usually raised to 72 °C (sometimes 68 °C) for the DNA polymerase enzyme to catalyze the template-directed syntheses of new double-stranded DNA molecules that are identical in sequence to the starting material. Hence, in a typical PCR assay, three temperature-controlled steps can be discerned, which

are repeated in a series of 25 – 50 cycles. One of the most significant temperatures in the PCR protocol is the annealing temperature which is defined by the primer pairs (forward and reverse) design (structure). In addition, the reaction PCR-mix consists of:

- A PCR-buffer (containing Tris-HCl, KCl, $MgCl_2$)
- A thermostable to single-standed DNA-polymerase, which adds nucleotides to the 3´-end of a primer annealed to single-stranded DNA (ssDNA)
- Four deoxyribonucleotide triphosphates [dNTPs]: dATP, dCTP, dGTP, dTTP
- Two oligonucleotide primers
- Template DNA

In the present study all amplifications were performed in an Eppendorf Mastercycler® Thermal Cyclers with a heated lid.

2.3.1 Protocol for PCR

➢ PCR mix

For all primer pairs, the PCR amplification was performed in a 30 µL volume. The following mix was adopted initially for all the primer pairs (genes) of Tables 1 – 3 (Deliverable 9), while specific optimisation was applied where needed.
PCR-mix contained:

10 mM Tris-HCl (pH 9.0),
2 mM $MgCl_2$,
1x Taq buffer
1.25 u Taq (GO Taq - Promega®)
0.4 µM each of forward and reverse primers (For Gluc_C12 2_1405 each of the primer had concentration 0.5 µM),
2 µL (~20 ng) of genomic DNA.

➢ Thermal cycling

For all primer pairs the same PCR-program (thermal cycling) was adopted, but with the relative annealing temperature for each primer pair (gene); optimisation was applied where needed. The thermal cycling for genes consisted of:

Step 1: 95°C for 5 min (denaturation)
Step 2: 80°C for 4 min (enzyme addition)
Step 3: 35 cycles
    94°C for 1 min
    Tan* for 1 min (annealing temperate)
    72°C for 1 min
Step 4: 72°C for 30 min (final extension)
Step 5: 16°C for ever
(Tan*: 60 °C was retained for all gene fragments apart from 2_1405 in which the hybridization temperature was 65 °C)

2.4 Gene sequencing

PCR products (from the above PCR reactions) were gel purified using the QIAquick® Gel Extraction Kit (Qiagen 2008). The amplification products served as template for the sequencing reactions based on the dideoxy-mediated chain termination method (Sanger et al. 1977). The BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) that was used, with the sequencing thermal profile described below:

Step 1: 96 °C for 1 min

Step 2: 35 cycles

96 °C for 10 sec

45 °C for 10 sec

60 °C for 40 min

Assessment of gene sequencing was carried out on the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems).

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