Report on DNA analysis to detect the possible occurrence of freshwater pearl mussels in the rivers Ammerån, Hemlingsån, Moälven, Öreälven, Rörströmsälven, Saxån and Vajbäcken (Sweden)

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Ecostreams for LIFE

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1. Objective

The “Ecostreams for LIFE” project aims at improving the conservation and ecological status of several aquatic habitats and species in six Natura 2000 sites using a holistic catchment approach. The habitats targeted are Fennoscandian natural rivers and water courses of plain to montane levels. Key species of conservation comprise the freshwater pearl mussel (*Margaritifera margaritifera*), Atlantic salmon (*Salmo salar*), otter (*Lutra lutra*), small liverwort (*Scapania carinthiaca*), noble crayfish (*Astacus astacus*) and sculpin (*Cottus gobio*). Within this project, one objective of the Technical University of Munich (TUM) is the detection of *Margaritifera margaritifera* populations by applying an environmental DNA (eDNA) approach.

2. Material and Methods

2.1 Sampling and DNA extraction

In September and October 2021, water samples were taken from six selected rivers (and one additional spot at a tributary (Vajbäcken) of the Öreälven) in Sweden in order to detect the possible occurrence of freshwater pearl mussels (*Margaritifera margaritifera*) by subsequent eDNA analysis. Water samples were collected from the following rivers: Ammerån, Hemlingsån, Moälven, Öreälven, Rörströmsälven, Saxån, and Vajbäcken (one spot) (**Table 1**, **Figure 1**,**2,3** and **4**).

Sampling locations were pre-defined on a map, with an average flow distance of 3 km between sites, depending on their accessibility. All rivers were sampled in upstream direction starting from the mouth of the river. Due to road conditions and for safety reasons, several pre-defined sampling locations were relocated on-site or had to be skipped. At each sampling site, three independent water samples of 0.5 l were taken from the free-flowing water with the help of a beaker attached to a pole (**Figure 1**). To avoid cross contamination between samples, a new

sterile plastic bag was placed into the beaker for every sample (Please see report: “Geist and Kuehn (2022) Short report on taking water samples from selected rivers in Sweden for subsequent eDNA analysis to detect the possible occurrence of freshwater pearl mussels”). Each water sample was then drawn through a glass fiber filter (Macherey & Nagel, diameter 2.5 cm, pore size 0.4 µm) on-site with a peristaltic pump (Vampir Sampler, Bürkle, Bad Bellingen). The filters were then transferred to 1.5 ml screw top vials, saturated with RNAlater solution (Thermo Fisher Scientific, Darmstadt) and stored in a refrigerator. The entire sampling procedure was also demonstrated by J. Geist, R. Kuehn and B. Stoeckle and jointly discussed with the project participants from Sweden during an on-site project meeting end of September 2021.

Figure 1: Water sampling directly from the free-flowing water (left) and three glass fiber filters after water sampling (spot R004, Rörströmsälven) (right).

DNA from filters was extracted with the DNeasy Blood and Tissue Kit Tissue kit (Qiagen, Hilden, Germany). To monitor possible contamination from reagents and equipment, filters treated with deionized water were included in the extraction process. All extractions were stored at –20°C until further processing.

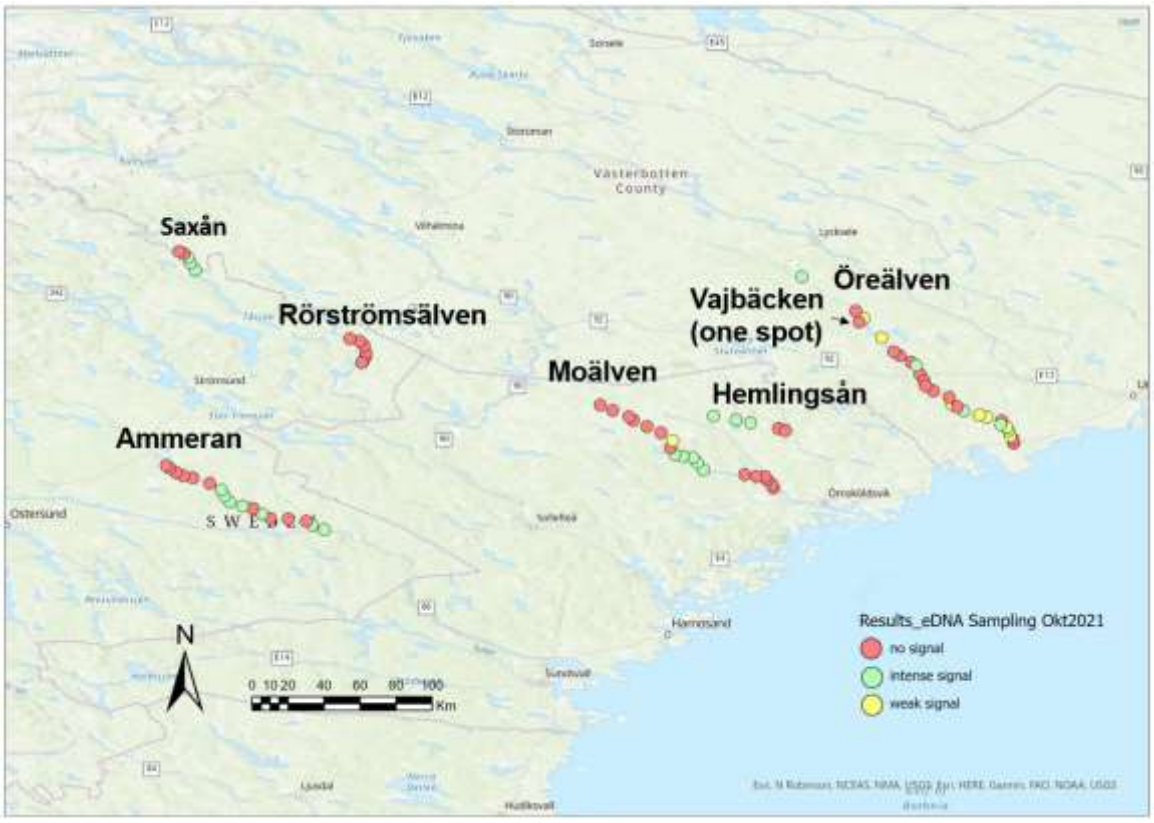

Figure 2: Geographic location of samplings spots at all sampled rivers: Ammerån, Hemlingsån, Moälven, Öreälven, Rörströmsälven, Saxån, and Vajbäcken (one spot)

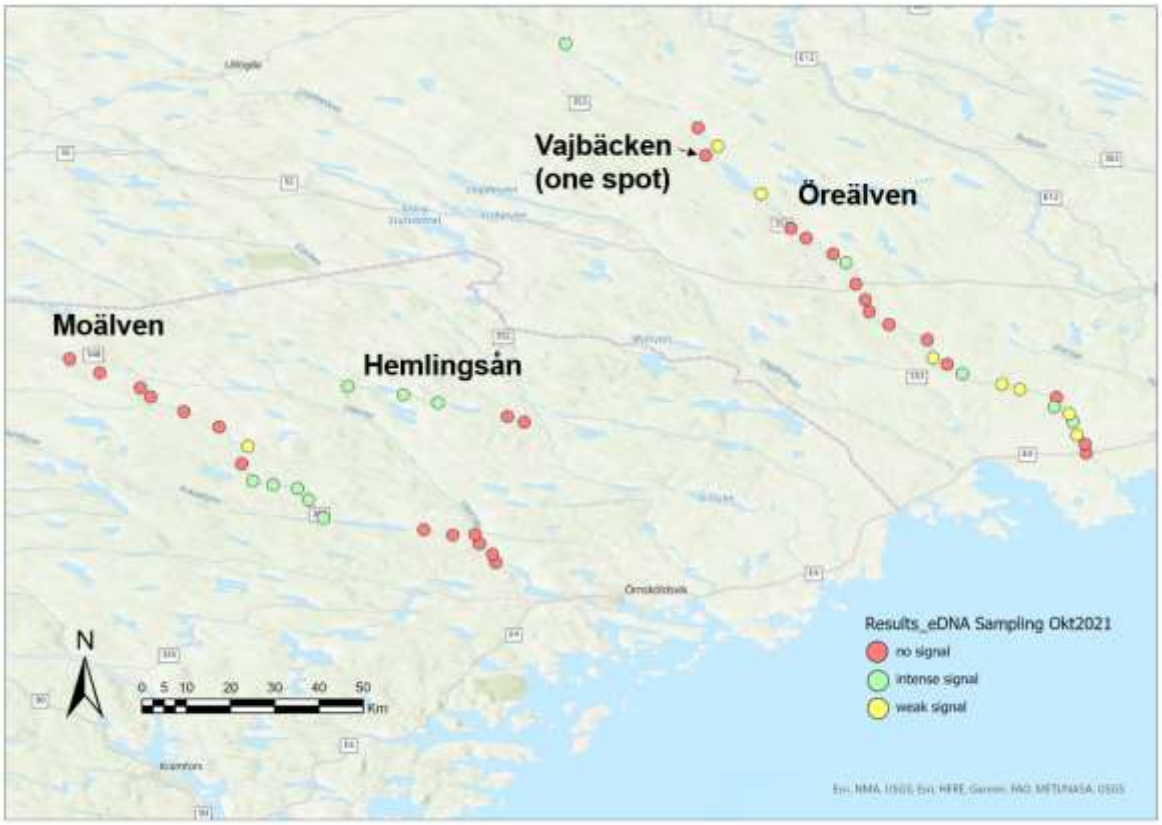

Figure 3: Geographic location of samplings spots at rivers Hemlingsån, Moälven, Öreälven and Vajbäcken (one spot)

Figure 4: Geographic location of samplings spots at rivers Ammerån, Rörströmsälven, and Saxån

2.2 eDNA analysis (qPCRs)

In order to minimize contamination, the DNA and PCR handling procedures used in this project were according to those for ancient DNA (aDNA) as described in Hofreiter et al. (2001). For all qPCR assays the species-specific primers MarMa_16S2.1 5'- GCAACAC-GGAAAACCCCTG -3' (forward) and MarMa_16S1.2 5'- GGCTGCGCTCATGTGAATTA -3' (reverse) were used to amplify a 16S fragment of 132 bp (Stoeckle et al. 2016). Amplifications were performed in triplets with inhibition-reducing qPCR assays (see Stoeckle et al. 2017 and Stoeckle et al. 2021) on an Agilent AriaMx Real-Time PCR System (Agilent Technologies Inc., Santa Clara, CA, USA) in 15 µL reactions consisting of 0.3 µL of each primer, 3.0 µL EvaGreen dye (Biotium, Tartu, Estonia), 7.5 µL Multiplex PCR Plus (Qiagen, Hilden, Germany) and 2.0 µL template (extracted DNA from all processed filters, including those treated with deionized water and reference samples). After an initial denaturation (5 m at 95 °C), the

qPCR was carried out in 40 cycles (30 s at 95°C, (denaturation) 30 s at 57 °C (annealing), 30 s at 72 °C (elongation) with a final continuous fluorescence acquisition (65 °C to 95 °C) for the melting curve analysis. For each PCR, one reaction containing no template was performed to monitor any possible contamination of PCR reagents. From each extracted water sample, three PCR replicates were prepared following the qPCR protocol mentioned above. In every qPCR run, four reference samples (DNA templates with 1 ng, 100 pg, 10 pg and 100 fg) were included in order to assess fluorescence signals and melting curves of extractions. DNA for reference templates originates from a previous project on pearl mussel genetics (Geist and Kuehn 2008). By using the Aria software (Agilent Technologies Inc.) PCR reactions were scored positive when a well-defined melting curve occurred and the fluorescence signal was above the calculated threshold (Cq-value), based on signals of reference samples. The Cq-value is the PCR cycle number at which a sample's reaction curve intersects the threshold line. This value tells how many cycles it took to detect a real signal from the samples. The lower this value, the higher the amount of target DNA.

3. Results and implications

In total, 79 spots were sampled at seven rivers. Consequently, 237 filters (79x3) were processed. At 31 spots (39%), DNA from *Margaritifera margaritifera* was detected: Ammerån (7), Hemlingsån (3), Moälven (6), Öreälven (12), Rörströmsälven (0), Saxån (3) and Vajbäcken (0). The complete information on eDNA detections is presented in **Figures 2, 3,4** and **5** as well as in **Table 1**). The detection of DNA is a strong indication that pearl mussels are present upstream of these 31 spots. Negative results do not necessarily exclude that the target species is not present since depending on ambient environmental conditions, DNA binding or degradation can vary among sites (Stoeckle et al. 2017). These findings have the following implications: When stream restoration work is being carried out in stretches with a strong positive eDNA signal, then special care should be taken to avoid harming the existing population by the work carried

out in the river, e.g. by visual inspection of the sites. On the other hand, sites sampled at the starting point of the project may be revisited after re-stockings with mussels or to evaluate the success of the restoration work. In this case, a switch from an originally absent signal to a positive detection, or an increase in the signal strength would both indicate success of the measures.

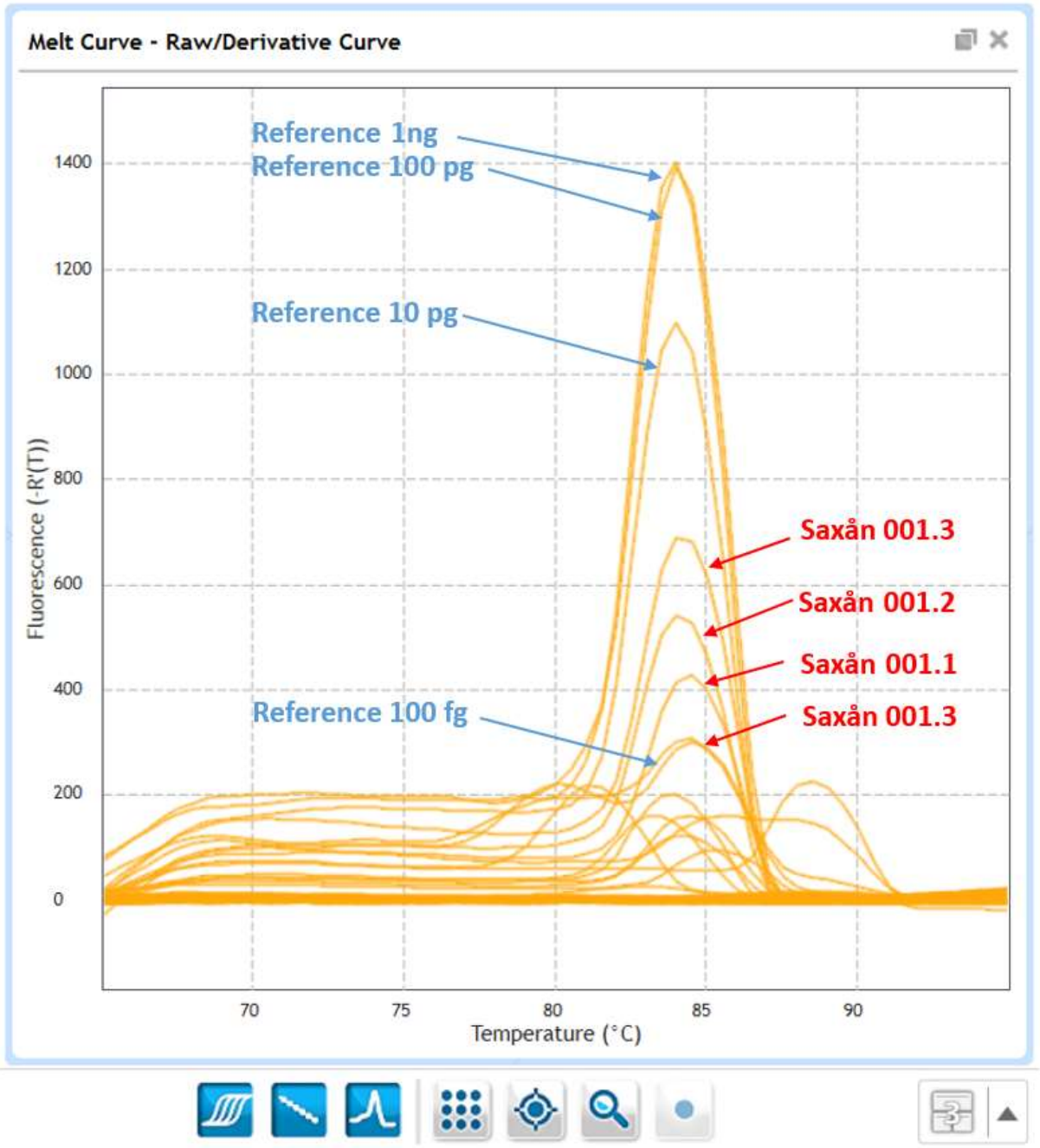

Figure 5: Example for a melting curve analysis by using the Aria software (Agilent Technologies Inc.) with four reference samples (DNA templates with 1ng, 100 pg, 10 pg and 100 fg, in blue) and three replicates per spot S001 to S005 (Saxån) and R001 to R006 (Rörströmsälven). Sampling spots with positive DNA signals are highlighted in red. Samples were scored positive when a well-defined melting curve occurred matching with melting curves of reference samples.

Table 1: Table 1 presents IDs, X- any Y-coordinates and eDNA analysis result of sampling spots at rivers Ammeran, Hemlingsån, Öreälven, Rörströmsälven, Saxån and Vajbäcken. Cq-values refer to lowest value detected at the specific spots based on three eDNA analysis repetitions. (NoDet = no detection)

Stream/Sampling spots	Cq-value	X-coordinate	Y-coordinate
Ammeran			
A001	27.77	16.2217614	63.1558315
A002	28.29	16.1609804	63.1789183
A003	NoDet	16.1309049	63.1982032
A004	NoDet	16.0413833	63.209263
A005	NoDet	15.9527063	63.2115072
A006	32.94	15.9109411	63.2339294
A007	NoDet	15.8654682	63.2621429
A008	38.44	15.8144351	63.2734164
A009	33.37	15.750826	63.2945797
A010	34.93	15.7230102	63.3239827
A011	35.14	15.7057712	63.3545352
A012	NoDet	15.6488181	63.3867462
A013	NoDet	15.5655425	63.4153524
A014	NoDet	15.5179486	63.4239891
A015	NoDet	15.4808129	63.441482
A016	NoDet	15.4511141	63.4624694
A017	NoDet	15.4325446	63.4746428
Hemlingsån			
H001	32.38	18.277557	63.705395
H002	32.14	18.348557	63.689358
H003	35.15	18.1654792	63.7226715
H004	NoDet	18.489073	63.6614
H005		18.523521	63.65046
Moälven			
M002	NoDet	18.4654485	63.3651632
M003	NoDet	18.4587489	63.3813206
M004	NoDet	18.4328067	63.4031421
M005	NoDet	18.4233872	63.4222833
M006	NoDet	18.3778761	63.4208618
M007	NoDet	18.3192654	63.4312455
M010	37.7	18.1150401	63.4548115
M011	35.7	18.0858226	63.4919399
M012	34.43	18.0625671	63.5157428
M013	34.13	18.0131006	63.5228866
M014	34.39	17.9718691	63.5304918
M015	NoDet	17.9493182	63.5656105
M016	38.93	17.9619704	63.6005818
M017	NoDet	17.9035931	63.6401446
M018	NoDet	17.8321347	63.6714325
M019	NoDet	17.7646074	63.7016214
M020	NoDet	17.7430032	63.7193811
M021	NoDet	17.6613015	63.750147
M022	NoDet	17.5995063	63.7782429

continuation Table 1

Stream/Sampling spots	Cq-value	X-coordinate	Y-coordinate
Öreälven			
OE001	NoDet	19.6645535	63.5874064
OE002	NoDet	19.662973	63.605435
OE003	38.85	19.6458319	63.6242753
OE004	33.89	19.6382669	63.651499
OE005	39.51	19.6307077	63.6667079
OE006	NoDet	19.6044663	63.6999934
OE007	39.64	19.5298555	63.7172421
OE008	39.95	19.4934685	63.727067
OE009	35.46	19.4132032	63.7484755
OE010	39	19.3541815	63.7800759
OE012	NoDet	19.3421529	63.8180256
OE013	NoDet	19.2638837	63.848675
OE015	NoDet	19.1968219	63.9308248
OE016	NoDet	19.1506448	63.9918233
OE017	NoDet	19.096167	64.0240254
OE018	NoDet	19.0651735	64.0437071
OE020	38.94	19.0046838	64.1138294
OE023	39.23	18.9166977	64.210708
OE025	NoDet	18.8761327	64.248224
OE026	37.34	19.1770281	63.974928
OE027	NoDet	19.2159131	63.8985556
OE028	NoDet	19.2241553	63.874387
OE031	NoDet	19.3822659	63.7680125
OE032	35.44	19.5998797	63.680977
OE033	28.26	18.607313	64.41905
Rörströmsälven			
R001	NoDet	16.4114391	63.9922385
R002	NoDet	16.426015	64.0176724
R003	NoDet	16.4303876	64.0359964
R004	NoDet	16.4193708	64.0740345
R005	NoDet	16.3994112	64.0966922
R006	NoDet	16.3496968	64.1096636
R007	NoDet	16.407955	63.990498
Saxån			
S001	31.98	15.577962	64.45339
S002	33.No-Det3	15.553148	64.48771
S003	33.64	15.542184	64.514755
S004	NoDet	15.520835	64.53208
S005	NoDet	15.496628	64.54454
Vajbäcken			
VA001	NoDet	18.891842	6.419226

4. Literature

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