REPORT ON THE EXPERIMENT:

"Storage methods of mixed pollen samples collected from traps: impact on botanical identification and estimation of relatives abundances as determined by ITS2 metabarcoding"

by

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The INSIGNIA project has relied on the collaboration of Citizen Scientists (CS), who have collected a large number of pollen samples in 2019 and 2020, making it possible an ambitious environmental study at the European level of using bee colonies for pesticide monitoring. This valuable collaboration is helping answering scientific questions on wide temporal and geographical scales that would otherwise be difficult to address. To facilitate storage at the CS premises of a large number of pollen samples collected across the bee active season, while at the same time assuring sample integrity for downstream molecular analyses, here we compared four different storage methods. The objective of this experiment was to assess whether the method of storing freshly collected pollen for long-time periods would affect botanical identification and relative abundances of mixed pollen samples, as determined by ITS2 metabarcoding, The ultimate goal of this experiment is to facilitate future Citizen Science projects by finding cheaper and easier methods for long-term storage of pollen samples that do not compromise the accuracy of downstream laboratorial analyses. The four different methods compared in this study for storing freshly collected pollen were: (i) in 96% ethanol, (ii) at -20ºC, (iii) desiccation at room temperature, (iv) desiccation with silica. Pollen storage, and later transportation between the CS premises and the analytical laboratories, is more expensive for samples placed in 96% ethanol or maintained frozen at -20° C than for samples treated by the methods involving desiccation.

METHODS

Pollen samples were collected from traps placed in front of beehives for < 1 day in two apiaries (one in Denmark and one in Austria) between August and September of 2020 (Table 1). After collection, pollen was homogenized and then split into replicates of 5g each. The storage methods consisted of:

1. ethanol: pollen was immersed in 96% ethanol and stored at room temperature;
2. silica: pollen was placed inside a porous tea bag and stored with 12g of silica; The silica was dried at 80ºC for two hours before the storage to assure homogenous conditions;

3. room temperature: pollen was placed on a fine gauze/filter-paper and dried at room temperature for one week and then was placed in vials and stored at room temperature;
4. frozen: pollen was stored at -20ºC soon after sampling.

All the samples, apart from the frozen ones, were stored at room temperature in the dark, to avoid damage from UV light, for >3 months until DNA extraction. The sample sizes per method, country, and sampling dates are shown in Table 1.

Table 1. Sample sizes for each storage method, country and sampling date.

Country	Sampling date	Ethanol	Silica	Room temperature	Frozen	Total
Denmark	11/08/2020	10	10	10	10	40
	05/09/2020	3	3	3	3	12
Austria	10/09/2020	3	4	4	3	14
	15/09/2020	5	6	6	5	22
Total		**21**	**23**	**23**	**21**	**88**

Botanical identification of the 88 samples was performed by DNA metabarcoding using high-throughput sequencing (HTS) with the nuclear barcoding marker ITS2 (internal transcribed spacer 2 regions of nuclear ribosomal DNA). DNA was extracted from ~50 mg of pollen using the NucleoSpin Food Kit Macherey-Nagel (Düren, Germany), according to manufacturer's instructions. DNA extracts were assessed for concentration and quality using a SPECTROstar® Nano (BMG Labtech, Ortenberg, Germany). DNA extracts were PCR-amplified using the universal primers ITS-S2F (Chen et al. 2010) and ITS-S4R (White et al. 1990), as part of an oligo scaffold that incorporates the MiSeq-specific adapters and the indexes. Library preparation for HTS was performed using a dual-indexing approach. The pollen samples were sequenced on the Illumina MiSeq platform using 2×250 cycles v2 chemistry. Analysis of sequence reads and taxa assignments were performed using an updated ITS2 reference database.

RESULTS

The results for the quality and concentration of the DNA extracts obtained with the four storage methods are shown in Figures 1 and 2. Significantly different DNA concentrations (P-value<0.05; Dunn test) were observed between ethanol and room temperature (Denmark and Austria) and ethanol and freezing (Denmark).

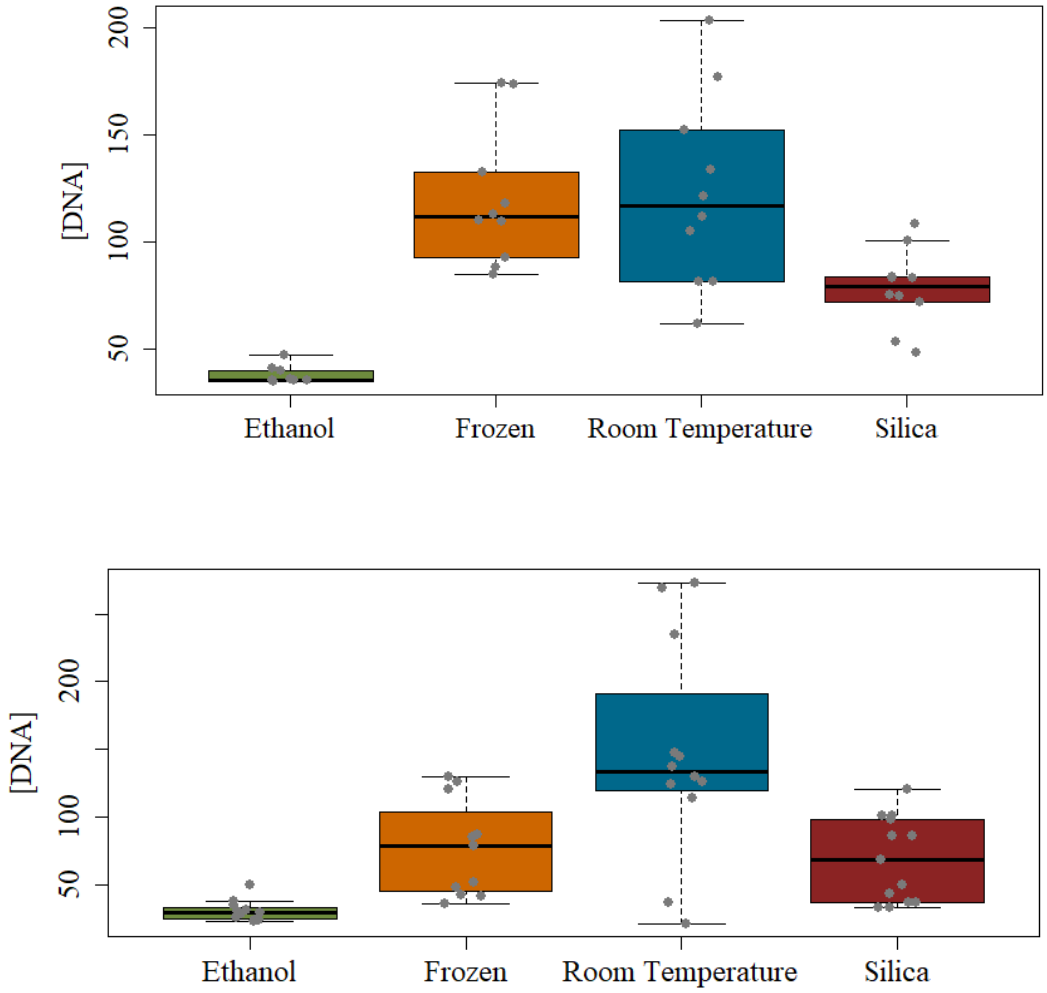

Figure 1. DNA concentrations obtained with the four storage methods for samples collected in Denmark (top) and Austria (down).

Despite the greater variation and poorer A260/280 absorbance ratio values for the room temperature method, the Dunn test did not detect significant differences (P>0.055) among storage methods for DNA quality (Figure 2).

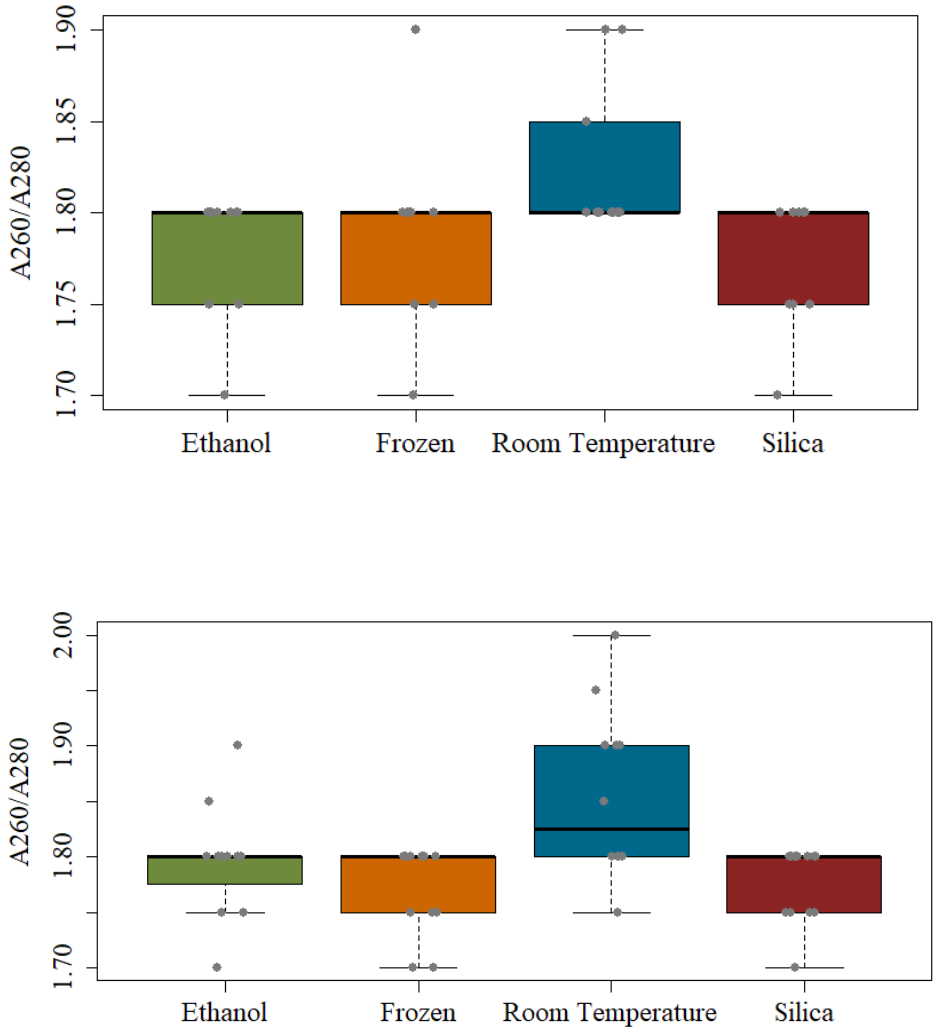

Figure 2. DNA quality (as measured by the A260/280 absorbance ratio) obtained with the four storage methods for samples collected in Denmark (top) and Austria (down). The best DNA quality is obtained when the 260/280 is ~1.8.

The means and standard deviations of the relative abundances estimated from the 88 mixed pollen samples, identified at the family level by the ITS2 metabarcoding procedure, are shown for each storage method in Table 2, for Denmark, and Tables 3,4, and 5 for the three sampling events in Austria. Consistent with the results obtained for quality and concentration, the relatives abundances

are very similar and do not statistically differ among the four storage methods (P-value > 0.99, Kruskal-Wallis test) in any of the sampling events (Tables 2-5). Altogether, the results obtained in this experiment suggest that the methods involving desiccation, which are cheaper than ethanol and freezing, can be used by the CS for long-term pollen storage for downstream applications involving DNA metabarcoding. Given that relative humidity at room temperature may vary greatly across countries and seasons, we recommend using the silica storage method.

Table 2. Relative abundance (mean and standard deviation, SD) at family level for each storage method in the Danish samples.

Family	**Ethanol**		**Silica**		**Room temperature**		**Frozen**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Family 1	0.2238	0.0224	0.2404	0.0141	0.2131	0.0224	0.2358	0.0170
Family 2	0.1956	0.0103	0.1919	0.0075	0.1908	0.0103	0.1932	0.0130
Family 3	0.1721	0.0227	0.0964	0.0174	0.0994	0.0251	0.1223	0.0212
Family 4	0.1155	0.0175	0.1539	0.0222	0.1503	0.0193	0.1506	0.0118
Family 5	0.0445	0.0185	0.0101	0.0063	0.0235	0.0190	0.0175	0.0151
Family 6	0.0128	0.0216	0.0039	0.0065	0.0015	0.0047	0.0019	0.0059
Family 7	0.0508	0.0106	0.0728	0.0128	0.0537	0.0114	0.0705	0.0101
Family 8	0.0179	0.016	0.0363	0.0203	0.0245	0.0257	0.0248	0.0117
Family 9	0.0380	0.0122	0.0414	0.0194	0.0343	0.0131	0.0363	0.0116
Family 10	0.0266	0.0201	0.0158	0.0126	0.0374	0.0143	0.0252	0.0156
Family 11	0.0659	0.0353	0.0482	0.0313	0.1019	0.0463	0.0543	0.0285
Family 12	0.0042	0.0133	0.0056	0.0091	0.0153	0.0194	0.0068	0.0112
Family 13	0.0225	0.0131	0.0558	0.0149	0.0368	0.0165	0.0416	0.0137
Family 14	0.0067	0.0095	0.0111	0.0123	0.0011	0.0036	0.0096	0.0093
Family 15	0.0031	0.0051	0.0093	0.0126	0.0144	0.0180	0.0097	0.0137
Family 16	0	0	0.0070	0.0148	0.0020	0.0063	0	0

Table 3. Relative abundance (mean and standard deviation, SD) at family level for each storage method in the Austrian samples collected at 05/09/2020.

Family	Ethanol		Silica		Room temperature		Frozen	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Family 17	0.3345	0.0102	0.3127	0.0246	0.2808	0.0350	0.2990	0.0592
Family 3	0.2002	0.0241	0.1153	0.0793	0.1084	0.0144	0.1223	0.0320
Family 14	0.1500	0.0094	0.1404	0.0259	0.1586	0.0141	0.1479	0.0069
Family 4	0.1334	0.0074	0.1778	0.0036	0.1825	0.0176	0.1655	0.0128
Family 1	0.0989	0.0127	0.1777	0.0306	0.1478	0.0247	0.1736	0.0025
Family 10	0.0210	0.0008	0.0125	0.0118	0.0160	0.0142	0.0184	0.0191
Family 18	0.0311	0.0147	0.029	0.0149	0.0541	0.0119	0.0318	0.0125
Family 19	0.0051	0.0088	0.0067	0.0058	0	0	0.0090	0.0064
Family 13	0.0097	0.0077	0.0087	0.0075	0.0094	0.0163	0.0058	0.0101
Family 20	0	0	0	0	0.0048	0.0042	0.0022	0.0038
Family 8	0.0160	0.0142	0.0192	0.0121	0.0376	0.0179	0.0244	0.0078

Table 4. Relative abundance (mean and standard deviation, SD) at family level for each storage method in the Austrian samples collected at 10/09/2020.

Family	Ethanol		Silica		Room temperature		Frozen	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Family 17	0.3780	0.0745	0.3864	0.0610	0.3731	0.0400	0.3469	0.0173
Family 3	0.1479	0.0264	0.1245	0.0080	0.1169	0.0352	0.1389	0.0199
Family 14	0.0759	0.0227	0.0914	0.0214	0.0506	0.0118	0.0659	0.0127
Family 4	0.1568	0.0056	0.1648	0.0126	0.1634	0.0198	0.1736	0.0120
Family 1	0.1170	0.0158	0.1426	0.0220	0.1564	0.0093	0.1296	0.0073
Family 10	0.0088	0.0086	0.0074	0.0086	0.0027	0.0031	0.0095	0.0070
Family 18	0.0207	0.0078	0.0115	0.0082	0.0340	0.0102	0.0175	0.0043
Family 19	0	0	0	0	0.0008	0.0015	0	0
Family 13	0.0008	0.0014	0	0	0.0054	0.0109	0.0083	0.0144
Family 20	0.0310	0.0031	0.0193	0.0057	0.0324	0.0042	0.0340	0.0022
Family 8	0.0167	0.0015	0.0027	0.0036	0.0096	0.0035	0.0026	0.0046
Family 21	0.0465	0.0404	0.0454	0.0493	0.0441	0.0300	0.0733	0.0287
Family 11	0	0	0.0042	0.0083	0.0106	0.0193	0	0

Table 5. Relative abundance (mean and standard deviation, SD) at family level for each storage method in the Austrian samples collected at 15/09/2020.

Family	Ethanol		Silica		Room temperature		Frozen	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Family 17	0.2963	0.0056	0.3352	0.0312	0.3126	0.0114	0.3029	0.0279
Family 3	0.2030	0.0233	0.1571	0.0298	0.1498	0.0142	0.1595	0.0264
Family 14	0.0297	0.0347	0.0153	0.0175	0.0270	0.0251	0.0100	0.0224
Family 4	0.0999	0.0158	0.1396	0.0104	0.1152	0.0196	0.1388	0.0154
Family 1	0.0810	0.0118	0.0876	0.0277	0.0960	0.0227	0.0988	0.0346
Family 10	0.0197	0.0180	0.0060	0.0065	0.0231	0.0293	0.0261	0.0264
Family 18	0.0288	0.0135	0.0275	0.0159	0.0432	0.0114	0.0350	0.0073
Family 19	0	0	0	0	0	0	0	0
Family 13	0	0	0.0068	0.0152	0.0034	0.0075	0.0039	0.0088
Family 20	0.2165	0.0189	0.1854	0.0152	0.2031	0.0080	0.2138	0.0038
Family 8	0.0087	0.0120	0.0077	0.0115	0.0148	0.0176	0.0112	0.0119
Family 22	0.0076	0.0169	0.0201	0.0311	0	0	0	0
Family 21	0.0088	0.0197	0	0	0	0	0	0
Family 2	0	0	0.0117	0.0147	0.0115	0.0081	0	0
Family 12	0	0	0	0	1.46E-05	3.26E-05	0	0
Family 23	0	0	0	0	1.46E-05	3.26E-05	0	0
Family 5	0	0	0	0	0.0001	0.0002	0	0
Family 11	0	0	0	0	4.37E-05	0.0001	0	0
Family 9	0	0	0	0	2.92E-05	0.0001	0	0
Family 24	0	0	0	0	2.92E-05	0.0001	0	0