

1 **Combining the resurrection approach with transplant experiments to investigate**
2 **adaptation of plant populations to environmental change**

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20

21 **Abstract**

22 Recent climatic changes, such as more frequent droughts and heatwaves, can lead to rapid
23 evolutionary adaptations in plant populations. Such rapid evolution can be investigated using
24 the resurrection approach by comparing plants raised from stored ancestral and contemporary
25 seeds from the same population. This approach has so far only been used in common garden
26 experiments, allowing to reveal genetic differentiation but not adaptation. In this study, we
27 performed a novel approach by testing for evolutionary adaptation in natural plant populations
28 using a resurrection study in combination with *in situ* transplantations. We cultivated seedlings
29 from ancestors (23-26 years old) and contemporary descendants of three perennial species
30 (*Melica ciliata*, *Leontodon hispidus* and *Clinopodium vulgare*) from calcareous grasslands in
31 the greenhouse and transplanted them back to their collection sites. In addition, we sowed seeds
32 of ancestors and descendants of two species (*L. hispidus* and *C. vulgare*) to the collection sites
33 in order to investigate germination rates. In transplanted *M. ciliata* seedlings, we observed lower
34 mortality, larger plant size and more leaves in descendants compared to ancestors. This
35 indicates that descendants are better adapted than ancestors to the current environmental
36 conditions, which proved to be exceptionally hot and dry during the study period. Descendants
37 of *C. vulgare* seedlings tended to be smaller and descendants of *L. hispidus* seedlings produced
38 less leaves compared to their ancestors in their contemporary environmental conditions. In
39 *C. vulgare* and *L. hispidus*, we found evolution towards faster germination, and especially
40 descendant seeds of *C. vulgare* were better adapted to the unfavourable conditions during the
41 experimental period. Concluding, we demonstrate that our novel approach to combine
42 resurrection ecology with transplant experiments is a promising avenue to rigorously test for
43 evolutionary adaptations in changing environments.

44 **1. Introduction**

45 Environmental conditions are rapidly changing since the last decades and will continue to
46 change in the future (IPCC, 2018). In North-western Europe, precipitation is expected to
47 decrease, especially in summer, and evapotranspiration will increase due to higher
48 temperatures. Together, these will lead to higher frequencies and intensities of droughts (Dore,
49 2005; Ruosteenoja et al., 2018; Samaniego et al., 2018; Spinoni et al., 2018). Given these
50 ongoing changes, understanding and predicting the capacity of natural plant populations to
51 evolve rapidly to such changing environmental conditions is a high priority in current and recent
52 research (Exposito-Alonso et al., 2018; Franks et al., 2018).

53 Over the last two decades, the resurrection approach has been applied as a powerful
54 method to study evolutionary changes of plant populations to recent global change (Franks et
55 al., 2018; Kooyers et al., 2021; Vtipil and Sheth, 2020). This approach consists of an
56 experimental design that uses seeds from a population collected before (ancestors) and after
57 (descendants) a potential selection pressure (*e.g.* consecutive years of drought, changes in
58 nutrient supply, etc.). Comparing phenotypes of these two generations in a common
59 environment may then reveal evolutionary changes (Franks et al., 2007). Recently, seeds
60 collected for storage in seed repositories have been successfully used in resurrection studies
61 (Ensslin et al., 2023; Everingham et al., 2021; Rauschkolb et al., 2022a, 2022b). Resurrection
62 studies convincingly showed that evolution of plant populations can occur rapidly within only
63 a few generations (Franks et al., 2007; Hamann et al., 2018; Thompson et al., 2013). Rapid
64 evolutionary changes have been documented in morphological, phenological, and physiological
65 trait changes in response to drought stress (Franks et al., 2007; Nevo et al., 2012; Sekor &
66 Franks, 2018; Thomann et al., 2015; Vigouroux et al., 2011). Many resurrection studies using
67 predominantly annual species reported evolution of earlier onset of flowering after a few years
68 of intense droughts (Franks et al., 2007; Nevo et al., 2012; Thomann et al., 2015). In some

69 species, seeds also emerged more rapidly and showed earlier flowering and smaller plant size
70 (Kulpa and Leger, 2013; Dickman et al., 2019; Sekor and Franks, 2018). These evolutionary
71 changes are consistent with a drought escape strategy involving rapid development to complete
72 the life cycle while resources are still available (Basu et al., 2016).

73 To date, the resurrection approach has mainly been applied in common garden
74 experiments located in a greenhouse, in *ex situ* experimental plots or in growth chambers
75 (Thomann et al., 2015; Vtipil & Sheth, 2020; Wooliver et al., 2020; Rauschkolb et al., 2022a,
76 2022b). As an interesting extension to resurrection studies, researchers have included
77 treatments or multiple environments to study whether, in addition to genetic differentiation of
78 trait means, the evolution of phenotypic plasticity has potentially been mediated by specific
79 environmental drivers (Blanquart et al., 2013; Rauschkolb et al., 2022a, 2022b). However, the
80 artificial conditions do not fully mirror those of the natural environment in which the
81 populations have evolved. For instance, temperature, water and nutrients are often much more
82 benign under controlled conditions (Poorter et al., 2016). Moreover, temporal dynamics of air
83 temperature and soil water availability, typical in natural environments, are very difficult to
84 realistically implement under experimental conditions (Poorter et al., 2016). Thus, these
85 experiments cannot deliver firm proof of adaptive evolution, since we cannot be sure how the
86 fitness of plants in *ex situ* cultivation relates to fitness in field conditions (Kawecki and Ebert,
87 2004). To convincingly demonstrate local adaptation, reciprocal transplant experiments remain
88 the gold standard (Kawecki & Ebert 2004, Blanquart et al 2013), and a logical step is therefore
89 to combine resurrection studies with *in situ* transplantations in the sites of population origins
90 (Franks et al. 2018), but to our knowledge no such studies have been published so far.

91 Reciprocal transplant experiments are a powerful tool to detect adaptation and
92 investigate fitness trade-offs by transplanting plants from different populations reciprocally to
93 all respective origins (Kawecki & Ebert, 2004; Fig. 1). This method can be partially applied to

94 the resurrection approach by transplanting ancestors and descendants to the exact location
95 where they were sampled (*in situ* transplantation; Fig. 1). By doing so, the strength of adaptation
96 of the ancestral and descendant populations to the current (but not to the past) environment can
97 be measured. Ensslin et al. (2023) already performed a similar method comparing plants in the
98 field from stored seed bank material, from *ex situ* cultivation and from a sampled wild
99 population close to the original population. However, their study did not focus on investigating
100 evolutionary change, but rather the effect of *ex situ* cultivation on germination and
101 establishment of plants reintroduced into the wild.

102 Here, we investigated recent adaptive evolution of three plant species from calcareous
103 grasslands in Belgium by conducting a resurrection study in combination with *in situ*
104 transplantations. We used ancestors sampled 23-26 years ago and contemporary descendants
105 of a population of *Melica ciliata*, *Clinopodium vulgare* and *Leontodon hispidus*. We
106 transplanted seedlings and sowed seeds from ancestors and descendants to the original sampling
107 location, we measured mortality, plant size and number of leaves on the seedlings, and we
108 observed germination of the seeds. We hypothesized that descendants show stronger adaptation
109 than their ancestors in terms of higher germination rate, better plant performance and lower
110 plant mortality under current conditions due to two decades of adaptation to environmental
111 changes. Finally, we discuss the conceptual integration of *in situ* transplantations into the
112 resurrection approach.

113

114 **2. Material and methods**

115 **2.1. Study species**

116 We selected three perennial plants that are typical species in calcareous grasslands and
117 widespread across Europe: *Melica ciliata* L. (Poaceae), *Clinopodium vulgare* L. (Lamiaceae)
118 and *Leontodon hispidus* L. (Asteraceae). *Melica ciliata* flowers in June, *C. vulgare* flowers

119 from July to September and *L. hispidus* flowers from June to October (Kühn and Klotz, 2002).
120 All selected species are hemicryptophytes and part of the floristic composition of semi-natural
121 dry grasslands. This habitat is considered as a conservation priority by the European
122 Commission ("Festuco-Brometalia"; EU code 6210: Semi-natural dry grasslands and scrubland
123 vegetation on calcareous substrates).

124 **2.2. Seed origin**

125 For each species, we chose one population in a calcareous grassland located in Belgian nature
126 reserves (Fig. 2, Table S1). The populations are relatively isolated and gene flow between other
127 populations should be minimal. The distance to the nearest population of the same species is
128 1.08 km for *M. ciliata*, 4.09 km for *C. vulgare*, and 1.86 km for *L. hispidus*. Seed material of
129 the ancestors were collected in the years 1992-1995 and seed material of the descendants in
130 2018-2020 depending on the species. *Clinopodium vulgare* and *L. hispidus* can reproduce in
131 the first year, while *M. ciliata* typically reproduces in the second year, which means there may
132 have been up to 23 generations for *C. vulgare*, 26 generations *L. hispidus* and up to 15
133 generations for *M. ciliata* between the collection years. Ancestral seed collections were
134 performed by the seed bank staff of Meise Botanic Garden (Belgium) for conservation
135 purposes. The botanical garden reported that the number of sampled individuals was maximized
136 to represent the population, but exact numbers were not recorded. All seeds were cleaned,
137 bulked, dried at 15 % relative humidity, and stored at -20 °C at the seed bank of the Meise
138 Botanic Garden. In the summer of 2018, seeds from 20-47 mother plants of *C. vulgare* and
139 *L. hispidus* were collected from the exact same populations (Table S1). Those seeds were
140 cleaned, bulked and then stored at 4 °C. Rauschkolb et al. (2022a) showed that genomic
141 relatedness of the ancestors and descendants of these populations is similar, which supports that
142 sampling procedures were comparable and a sufficient number of seeds was collected. Seeds

143 from *M. ciliata* were collected in 2020 by the Meise Botanic Garden using the same sampling
144 protocol as applied in 2018.

145 We obtained meteorological data from the Royal Meteorological Institute of Belgium
146 for the weather station “Dourbes”, which is located 3 km from the *M. ciliata* population and
147 11 km from the *C. vulgare* population, as well as from the Royal Netherlands Meteorological
148 Institute for the weather station “Maastricht”, which is located 14 km from the *L. hispidus*
149 population. The meteorological data contains daily maximum and mean temperature as well as
150 daily precipitation from 1966 until 2022. We calculated the mean and maximum temperature
151 and total precipitation for each month during the growth season from March to August of 2022
152 as well as the 30-year-average (1991-2021). These data were used to compare the weather
153 conditions during the year of study with the long-term average. Furthermore, we used the
154 homogenized data from the “Dourbes” weather station to calculate the temperature anomaly for
155 each year as the difference between its average annual temperature and the average temperature
156 from 1966 until 2020 (Fig. S2 B). Following Liao et al. (2020), we additionally calculated the
157 climatic water deficit (CWD) for this area, which is an estimate of drought stress in plants.
158 CWD is quantified as the amount of water by which potential evapotranspiration exceeds actual
159 evapotranspiration (Stephenson, 1998). Solar radiation data was retrieved from Hargreaves
160 (1994). The temperature anomaly steadily increased from 1966 until 2020 and mean
161 temperature in the seed collection years was 9.41 °C in 1992 (ancestors) and 10.25 °C in 2018
162 (descendants; Fig. 2S B). The average CWD steadily increased during the period from 1966 to
163 2020 and was 66 % higher in 2018 compared to 1992 (Fig. 2S A). The focal populations were
164 thus exposed to a progressively diminishing water availability in response to increasing
165 temperatures between sampling of ancestors and descendants.

166

167

168 2.3. Experimental design

169 To reduce environmental, maternal and storage effects (Franks et al., 2007), a refresher
170 generation of both ancestors and descendants (hereafter referred as ‘temporal origins’) was
171 grown under standardised greenhouse conditions (Rauschkolb, et al., 2022b). We sowed 200-
172 300 seeds of each temporal origin and 15 seedlings were randomly selected and cultivated for
173 each temporal origin. We kept the plants from the two temporal origins in separate cages to
174 prevent unintentional cross-pollination (Rauschkolb, et al., 2022b). Emerging flowers were
175 randomly pollinated by hand using pollen from individuals of the same temporal origin. Seed
176 production of *L. hispidus* was too low for the experiment, hence another refresher generation
177 was grown using the same protocol, but this time bumblebees (Natupol Seeds, Koppert GmbH)
178 were used for pollination. The refresher generation failed for *M. ciliata*, because the plants did
179 not flower, so for this species we used seeds without a refresher generation in our experiments.

180 In September 2021, refreshed seeds from 12 seed families per temporal origin of
181 *C. vulgare* and *L. hispidus* and from 14 seed families of *M. ciliata* descendants were sown in
182 trays with cultivation soil (Anzuchterde, Hawita Fruhstorfer Erde) to produce 14 seedlings per
183 seed family. For ancestors of *M. ciliata*, we sowed 100 random seeds from the ancestral seed
184 bulk, which resulted in 60 plants available for the transplantation. After germination, seedlings
185 were singled out and transplanted to 54-multitray-pots (Meyer KG) with standard potting soil
186 (Type T1b, Hawita Fruhstorfer Erde). The seedlings were kept in the greenhouse of the
187 “Wissenschaftsgarten” at Goethe University Frankfurt. Three days prior to transplantation
188 (October 2021) we measured plant size variables: height of the longest stem for *C. vulgare* and
189 *M. ciliata* and rosette diameter for *L. hispidus*. After the measurements the plants were moved
190 outdoors for cold acclimation. To avoid confusion with other individuals of the same species,
191 we set up a plot for transplantation in the vicinity of the original population where no natural
192 individuals were present. The distance from the plot to the edge of their natural population is
193 295 m for *M. ciliata*, 15 m for *C. vulgare* and 105 m for *L. hispidus*. The plots for *L. hispidus*

194 and *M. ciliata* were situated on flat ground while the plot for *C. vulgare* was situated on a slope.
195 To prevent herbivory and trampling by large grazers, each plot was fenced. We cut the standing
196 vegetation with a brushcutter to facilitate transplantations. The plots were divided into 12 blocks
197 for *C. vulgare* and *L. hispidus* and into 10 blocks for *M. ciliata*. The blocks were 1 m × 1.5 m
198 wide and contained 24 individual plants in 4 columns and 6 rows separated by 25 cm. Plants
199 were distributed among the blocks equally according to temporal origin and seed family, but
200 randomized within each block. In February 2022, we visited the plots to sow seeds from
201 *C. vulgare* and *L. hispidus*. We collected local soils within the plot and sieved it through a 5
202 mm mesh size. The sieved soil was used to fill six 54-multitray-pots (Meyer KG), which were
203 placed with their surface at ground level. For each seed family of ancestors and descendants,
204 we sowed ten seeds into twelve pots (replicates). The replicates were distributed equally among
205 the multitrays (blocks) and then randomized in each tray. After sowing, the trays were covered
206 with a fine white net curtain to prevent seed spillover and herbivory interference.

207 In the center of each plot, one data logger (iButton DS1923, Maxim Integrated) was
208 positioned to record air temperature and relative humidity every four hours over the whole
209 experimental period. Additionally, two data loggers (iButton DS1921G-F5, Maxim Integrated)
210 were buried 5 cm deep in the center of two opposing corner blocks to measure soil temperature
211 every 4 hours. Furthermore, in order to make sure that soil conditions were comparable, we
212 took four random 25 cm² soil samples from each transplantation plot and from within the native
213 population site at 10 cm depth. The four samples from each location were mixed together and
214 dried at 40°C for one week. The samples were sieved to < 2 mm, and 0.3–1g of sieved soil was
215 milled with the Mixer Mill MM400 (Retsch, Haan, Germany) for 60 seconds with 30 rounds
216 per second. To avoid contamination between samples, the sieving and milling tools were
217 cleaned between samples with an air-compressor and water. The chemical composition of the
218 samples was analyzed to determine the amount of fundamental minerals for plant development

219 (*i.e.*, plant-available P, K, S, Ca and Mg, total element content of P, K, S, Ca, total C, N and S),
220 as well as pH level and salinity (Table S4).

221 **2.4. Measurements in the field**

222 The plots were revisited in April 2022 and we measured the following: germination, mortality,
223 number of leaves and plant size. In July 2022, we only visited the *M. ciliata* plot due to time
224 constraints and recorded mortality and plant size. We visited all plots in August 2022 and
225 recorded germination, mortality, and plant size. In April 2023, we only recorded germination at
226 the *C. vulgare* and *L. hispidus* plots.

227 **2.5. Data analysis**

228 All analyzes were conducted using R (version 4.0.3, R Core Team, 2020). To compare
229 *M. ciliata* ancestors (60 plants, seed families unknown) and descendants (180 plants from
230 14 seed families), we created a completely random subset of the descendants consisting of
231 60 plants. No significant differences ($p < 0.05$) in plant size and number of leaves were found
232 between the randomly selected subset of descendants and the complete dataset (Table S2), and
233 we therefore used the subset for further analysis.

234 We applied generalized linear mixed-effects models (GLMM) with binomial family
235 implemented in the *lme4* package (Bates et al., 2015) to analyze differences in mortality
236 (response variable) between temporal origins (*i.e.*, ancestors vs. descendants). Temporal origin,
237 time (*i.e.*, the different times when plants were measured) and their two-way interaction were
238 included as fixed factors, and seed family (for data of *C. vulgare* and *L. hispidus*) and block as
239 random factors.

240 We used linear mixed-effects models (LMMs, *lme4* package) to test for differences in
241 morphological traits between temporal origins at several measurement times. Plant size and
242 number of leaves were included as response variables in separate models. For plant size, we
243 included temporal origin, time and their two-way interaction as fixed factors, and seed family

244 (for data of *C. vulgare* and *L. hispidus*), block and individual (to account for multiple
245 measurements over the same individual) as random factors. For number of leaves, the same
246 model was used, but we additionally included initial size as covariate to control for potential
247 differences in size of the transplanted seedlings.

248 We also analyzed whether died plants had a different plant size from plants that survived in
249 the previous measurement using LMMs. To this aim, we included plant size as response
250 variable and mortality at the next measurement, temporal origin and their two-way interaction
251 as fixed factors, and seed family (for data of *C. vulgare* and *L. hispidus*), block and individual
252 (for data of *M. ciliata*) as random factors. We used a logistic regression to confirm the results
253 of this model (GLMM, binomial family) with mortality as the response variable and time, plant
254 size at the previous measurement and their interaction as explanatory variables. We also
255 included seed family (*C. vulgare* and *L. hispidus*) and block as random factors.

256 In addition, we used generalized linear mixed-effects models (GLMM) with Poisson family
257 implemented in the *lme4* package (Bates et al., 2015) to analyze differences in the number of
258 germinated seeds (response variable) between temporal origins. Temporal origin, time and their
259 interaction were included as fixed factors and block, seed family nested in block, and individual
260 as random factors. We checked the model for zero inflation using the function
261 “check_zeroinflation” from the *performance* package (Lüdtke et al., 2021). The model for
262 *C. vulgare* was underfitting zeros (ratio of 0.80), hence we used a negative binomial GLMM
263 from the *lme4* package (Bates et al., 2015) instead.

264 In all LMMs, the assumptions of normality and homogeneity of variance of the residuals
265 were tested using the Shapiro-Wilk test and visually checked through plotting the fitted versus
266 residual values. If needed, the response variables were log- or square-root-transformed to meet
267 the parametric assumptions. Whenever time of measurement or the temporal origin \times time of

268 measurement interaction was significant, we applied post-hoc contrasts using Tukey's test from
269 the *emmeans* package (Lenth, 2021).

270

271 **3. Results**

272 In *M. ciliata* mortality did not differ between temporal origins in April 2022, but was
273 significantly higher in ancestors compared to descendants in July 2022 (55.0 % mortality in
274 ancestors and 20.0 % mortality in descendants, Fig. 3A) and in August 2022 (78.3 % *versus*
275 56.7 % mortality, respectively, Fig. 3A). Mortality was significantly affected by temporal origin
276 and time of measurement ($p < 0.001$, Table 1), but not by their interaction ($p = 0.804$, Table 1).
277 Plant size significantly differed depending on mortality at the next measurement ($p < 0.001$,
278 Table 1) and, vice versa, mortality differed depending on the plants size at the previous
279 measurement (Table S3), meaning that *M. ciliata* plants that would not survive until the next
280 census were generally smaller than the plants that survived (Fig. 4A). Moreover, plant size was
281 significantly affected by temporal origin, time of measurement and their interaction ($p < 0.001$,
282 Table 1). Descendants were bigger compared to the ancestors at all measurement times
283 (Fig. 3B). Furthermore, there were no significant differences in plants size of descendants
284 between October 2021 and April 2022, whereas ancestors grew significantly by 1.49 ± 0.12 cm
285 (Mean \pm Standard Error, Fig. 3B) during this time. In April 2022, descendants carried
286 6.65 ± 0.47 leaves, which was significantly more than ancestors which had 4.75 ± 0.34 leaves
287 ($p = 0.002$, Fig. 3C, Table 1).

288 In *C. vulgare*, mortality did not differ between temporal origins in April 2022 ($p = 0.339$,
289 Table 1) and reached 100% in August 2022 for both temporal origins (Fig. 3D). Plant size was
290 marginally significantly affected by mortality ($p = 0.092$, Table 1), but post-hoc tests did not
291 reveal significant differences between alive and dead plants ($p > 0.05$, Fig. 4B) and the logistic

292 regression found no significant effect of plant size on mortality ($p = 0.207$, Table S3). However,
293 plant size was significantly affected by time of measurement ($p < 0.001$, Table 1) and the
294 interaction of time of measurement and temporal origin ($p = 0.006$, Table 1). Plant size and
295 number of leaves were not significantly different between ancestors and descendants ($p > 0.05$,
296 Table 1, Fig. 3EF). From the seeds we sowed in the experimental plot, we recorded germination
297 neither in April 2022 nor in August 2022 regardless of the temporal origin (Fig. 5A). In April
298 2023, however, we recorded significantly more seedlings of descendants (3.69 ± 0.17 seedlings
299 per pot, Fig. 5A) compared to ancestors (3.31 ± 0.18 seedlings per pot, Fig. 5A, $p = 0.03$,
300 Table 2).

301 In *L. hispidus*, mortality did not differ between temporal origins ($p = 0.316$, Table 1)
302 and reached 100% in August 2022 for both temporal origins (Fig. 3G). Plant size tended to
303 differ between dead and alive plants ($p = 0.058$, Table 1) and plant size significantly affected
304 mortality according to the logistic regression ($p < 0.01$, Table S3). However, post-hoc tests did
305 not reveal significant differences between alive and dead plants ($p > 0.05$, Fig. 4C), but
306 surviving plants tended to be bigger than those that would die. Plant size was significantly
307 affected by temporal origin ($p = 0.013$, Table 1), time of measurement ($p < 0.001$, Table 1) and
308 their interaction ($p = 0.035$, Table 1). Descendants were 1.18 cm bigger than ancestors in
309 October 2021, but had the same size as ancestors in April 2022 (Fig. 3H). Number of leaves
310 was significantly higher in ancestors (7.77 ± 0.25 , Fig. 3I, $p = 0.002$, Table 1) compared to
311 descendants (7.03 ± 0.16 , Fig. 3I) in April 2022. Regarding the seed sowing experiment, there is
312 a significant interaction of temporal origin and time in terms of the number of recorded
313 seedlings ($p = 0.013$, Table 2). In April 2022 we recorded significantly more seedlings per pot
314 of descendants (0.43 ± 0.09 seedlings, Fig. 5B) compared to ancestors (0.22 ± 0.06 seedlings,
315 Fig. 5B, post-hoc test: $p = 0.0084$). At the next census in August 2022, no seedlings were
316 recorded for both descendants and ancestors, while in April 2023 new seedlings had emerged

317 but descendants (0.36 ± 0.07 seedlings, Fig. 5B) and ancestors (0.45 ± 0.10 seedlings, Fig. 5B)
318 did not significantly differ in this respect (post-hoc test: $p = 0.5955$).

319 **4. Discussion**

320 In this study, we incorporated transplantation experiments into the resurrection approach to
321 study adaptive evolution of three populations of different plant species to recent environmental
322 change. We detected evolutionary changes in all three study species. We found greater plant
323 size, more leaves and lower mortality in descendants of *M. ciliata* compared to their ancestors.
324 Descendants of *C. vulgare* tended to produce a lower plant size and slightly more germination
325 in field conditions compared to their ancestors. In *L. hispidus*, we found a larger plant size and
326 less leaves in the juvenile stage of the descendants compared to their ancestors. We also
327 observed a higher percentage of germinated seeds in *C. vulgare* and *L. hispidus* in descendants
328 compared to ancestors.

329 **4.1. Genetic differentiation between descendants and ancestors in their natural** 330 **environment**

331 In the first year of the experiment, the germination of the seeds we sowed in the field was very
332 low. We did not record any seedlings of *C. vulgare* in 2022, which was most likely driven by
333 the extremely low water availability during this year. Seeds of *L. hispidus* managed to germinate
334 in April 2022 and we recorded more descendant seedlings (4.3 % of seeds sown) compared to
335 ancestors (2.2 % of seeds sown). Thus, descendant seeds seem to be able to germinate faster or
336 need less water to germinate. However, all seedlings died before the next census in Autumn
337 2022 irrespective of temporal origin. Hence, in our experiment the faster germination of the
338 descendant seedlings did not lead to a drought escape (Basu et al., 2016), because all plants
339 died before they could reproduce. We have to consider, that our experimental setup does not
340 fully represent natural conditions. If the seeds would have germinated on the natural soil – i.e.
341 not in multitrays with local soil – in the natural vegetation – i.e. not clipped to facilitate the

342 transplantation –, the faster germination might have helped the seedlings to grow deeper roots
343 to reach soil layers with higher water content in a potentially more benign aboveground
344 microenvironment. In April 2023, some seeds of *L. hispidus* germinated, but there were no
345 significant differences in germination rate between descendants and ancestors. The conditions
346 in April 2023 were much more favourable compared to the previous year, but the germination
347 rate of *L. hispidus* was still similar to April 2022. This might be due to high seed mortality
348 during 2022 or mortality of seedlings that germinated in between our measuring visits.

349 In contrast, we recorded a high number of seedlings of *C. vulgare* in April 2023, with more
350 seedlings of descendants (36.9 % of seeds sown) compared to ancestors (31.3 % of seeds sown).
351 These germination rates are in the same range of what we have observed under greenhouse
352 conditions, which usually fluctuate around 45 %. The higher seedling count of the descendants
353 can have multiple causes. The population might have evolved a higher longevity in the dormant
354 state leading to more viable seeds (Dalling et al., 2011). Descendant seeds might also require
355 less water for germination or have more efficient water uptake to facilitate germination (Baskin
356 and Baskin, 2015). It is also possible that less descendant seedlings germinated and
357 subsequently died before we could document their germination. Even though we cannot
358 pinpoint the underlying cause with our data, the findings show that the descendant seeds are
359 better adapted to the contemporary environmental conditions.

360 Mortality rates were high for transplanted plants of all species in 2022, which impeded the
361 follow-up of the entire life cycle of the study species. This was very likely caused by the
362 extreme temperatures and drought (*e.g.*, soil temperatures were up to 50°C for *C. vulgare*;
363 Fig. S3H). These microclimatic conditions might have been exacerbated by cutting the standing
364 vegetation, which significantly reduced shade cover. In the growing season of 2022 (March to
365 August), the total precipitation in the experimental sites was up to 50 % lower compared with
366 the 30-year-average (Fig. S1). In addition, maximum temperatures reached 39.6 °C in July

367 2022, being on average 7°C above the maximum temperature of the previous 30 years
368 (Fig. S1). The descendants of *M. ciliata* survived these dry and hot conditions at a higher rate
369 compared to their ancestors, suggesting the evolution of higher drought tolerance or improved
370 avoidance strategies. Drought avoidance strategies can involve increased root growth,
371 xeromorphic leaf structures such as smaller and thicker leaves, higher stomatal density (Fang
372 and Xiong, 2015) whereas drought tolerance involves improved osmoregulation (Morgan,
373 1984).

374 We found higher plant size and more leaves in descendants of *M. ciliata*, which may
375 indicate greater acquisition and retention of resources as well as higher fitness. The higher
376 performance of descendants can be explained by evolution of adaptation to the current
377 environmental conditions over time (Pluess, 2013). In the case of *L. hispidus*, the initial size
378 (measured in October 2021) of the descendants upon transplantation was much higher
379 compared to the ancestors, but we could not detect any differences in the following spring.
380 Thus, the descendants of *L. hispidus* performed better under controlled and comparatively
381 unnatural conditions in the greenhouse, whereas they grew at a slower rate under field
382 conditions and produced less leaves than the ancestors. We can see a similar tendency for
383 *C. vulgare* regarding the interaction of origin with time of measurement for plant size. Here,
384 descendants tended to grow slower than ancestors, which was also previously found in the same
385 populations in a greenhouse study (Rauschkolb et al. 2022a). Reduced growth can be explained
386 as a response to dry conditions as it reduces water loss by transpiration and reduces resource
387 needs, and this explanation is supported by a multi-species experiment investigating ten
388 grassland species under variable water availability treatments demonstrating that smaller
389 seedlings have a higher survival rate (Harrison and LaForgia, 2019).

390 Despite slower growth of descendants compared to ancestors in *C. vulgare* and
391 *L. hispidus*, we could not detect differences in mortality between temporal origins, which may

392 be explained by the exceptional weather conditions during the growing season, since mortality
393 reached 100% in August 2022 for both temporal origins and species. We also observed that
394 larger plants tended to survive better overall, contradicting the notion that small plants are better
395 adapted to dry conditions (Olson et al., 2018). We therefore conclude that the observed reduced
396 plant size in descendants compared to ancestors seems to be a stress response rather than an
397 adaptation to reduce evapotranspiration.

398 Overall, our findings indicate genetic differentiation between ancestors and descendants
399 of our study populations in their natural habitat. Since the transplantation sites are very close to
400 the collection sites (15 – 295m) and since the soil conditions are similar (Table S4), we can
401 discuss the results with respect to evolutionary adaptation. Since we investigated rather isolated
402 populations, we can attribute trait differentiation to evolutionary change by natural selection on
403 standing variation rather than by immigration (Pluess, 2013). However, the population size is
404 rather small for all three populations and neutral genetic drift may have played a role in trait
405 differentiation. Moreover, the results for *M. ciliata* have to be interpreted with care, since we
406 did not successfully grow a refresher generation to reduce storage and maternal effects (Franks
407 et al., 2018). Another potential confounding factor can be the so-called “invisible fraction”, *i.e.*,
408 only a fraction of the seeds may have survived the storage conditions, which might be
409 genetically correlated with post-emergence plant traits (Weis, 2018). However, since the
410 germination rate of stored seeds was high in *M. ciliata* (100%) and *L. hispidus* (93%) and
411 intermediate in *C. vulgare* (45 %), the invisible fraction cannot have influenced the results of
412 *M. ciliata* and *L. hispidus* and the influence on *C. vulgare* might be small as well, given that
413 stored seeds had a similar germination rate as newly sampled seeds (40%).

414

415 **4.2. Timing and strength of selection pressure**

416 The mortality data, assessed at several points in time, allowed us to investigate the strength of
417 the selection pressure during different time periods. The highest selection during autumn-winter
418 (October – April) was experienced by *C. vulgare* (37.5 - 42.3 % mortality, Fig. 3D) followed
419 by *L. hispidus* (11.1 - 16.0 % mortality, Fig. 3G) and the lowest selection was found in for
420 *M. ciliata* (0 - 13.3 % mortality, Fig. 3A). All three species are hemicryptophytes with renewal
421 buds at ground level during winter (Niklas, 2008). The main causes of mortality in winter are
422 usually freezing temperatures (Stushnoff et al., 1984). *Clinopodium vulgare* might be more
423 exposed to the harsh weather compared to the other species, considering that the plants were
424 transplanted on a slope from which the potentially protective vegetation layer was removed.
425 Given the generally high mortality of all plants across species, any evolutionary changes over
426 the last 23-26 years that would have conferred improved drought tolerance were not sufficiently
427 strong to cope with the extreme conditions experienced in the study year. However, this
428 observation applies only to juvenile plants as their root system is smaller than that of adult
429 plants. In contrast, adult plants may have a deeper root system enabling them to survive under
430 these conditions (Basu et al., 2016) and selection for improved drought tolerance at the adult
431 stage may have been comparatively more successful.

432 Climatic predictions forecast more frequent extreme heatwaves and droughts in the next
433 years (Dore, 2005; Ruosteenoja et al., 2018), which may further threaten the establishment and
434 persistence of plant populations. This should be especially true if extreme conditions occur in
435 consecutive years, since natural population dynamics becomes disturbed due to lack of
436 recruitment and populations would have difficulties to recover from this (Crawley et al., 1990).
437 In the case of *M. ciliata*, the strength of selection pressure depended on the temporal origin.
438 The highest mortality increases for ancestors occurred between April and July 2022
439 (41.7 percentage points), and for descendants between July and August 2022 (36.7 percentage
440 points), indicating that ancestors experienced selection pressure much earlier than descendants.

441 These results support the idea that the population has evolved local adaptation to dryer
442 conditions over the last 26 years leading to lower mortality.

443

444 **4.3. Extending the resurrection approach by including *in situ* transplantations**

445 The resurrection approach is a powerful method to study contemporary evolution of plant
446 populations (Franks et al., 2018). It is constantly developed and applied in new contexts.
447 Previous studies focused mainly on how single fast-growing annual species responded to
448 experimental drought under controlled conditions (Hamann et al., 2021; Kooyers et al., 2021;
449 Lambrecht et al., 2020). Recent resurrection studies also used multiple species and included
450 treatments (*e.g.* herbivory, Rauschkolb et al., 2022b). However, these studies cannot prove
451 whether the observed evolutionary changes are adaptive to their habitat of origin. With our
452 current study, we propose and demonstrate in practice a novel methodological approach
453 applying *in situ* transplantations within a resurrection ecology framework. Transplanting
454 ancestors and descendants to the exact same location where they have been previously sampled
455 provides researchers the ability to observe evolutionary change in the environment where the
456 responsible selection pressures acted. In addition, seeds of ancestors and descendants can be
457 sown at the collection sites in order to investigate evolutionary change in germination rate and
458 seedling establishment. In our experimental demonstration, we found evidence of rapid
459 adaptation in the study species. Nevertheless, we should consider the nursing period of the
460 plants in the greenhouse prior to transplantation, which may prime ancestors and descendants
461 differently.

462 We see strong potential in combining the resurrection approach with *in situ*
463 transplantations and have further recommendations. First of all, we recommend monitoring the
464 plants at the study sites over multiple years, which is important for perennials, especially if
465 species need several years to flower, to gain a better insight into lifetime fitness. This can also

466 enhance the robustness of the results by revealing natural fluctuation as a result of phenotypic
467 plastic responses due to year-to-year variation in environmental conditions. Either the average
468 phenotype across multiple years or the phenotypic expression during specific years may elicit
469 plastic responses that reflect evolutionary adaptations. Secondly, we recommend the use of
470 more than two generations, ideally a series of generations, to investigate the natural fluctuation
471 in phenotypic expression across generations. This is important because it is often assumed that
472 evolutionary changes build up gradually over time whereas these could also be the result of
473 extreme selection events in single years (Gould and Eldredge, 1977). Thirdly, combining multi-
474 year monitoring with continuous data logging of climatic variables may allow inference of the
475 strength of selection induced by the environmental conditions over the different years. Finally,
476 we suggest to include multiple populations of the same species, if these are available, to allow
477 more general statements about the evolutionary potential of the species.

478

479 **5. Conclusion**

480 We investigated three perennial plant species using a novel approach that combined a
481 resurrection experiment with *in situ* transplantations. We found evidence for evolutionary
482 adaptations after 23 – 26 years of climate change in all species, but changes were most
483 prominent in *M. ciliata*. Here, we observed lower mortality and larger plant size of descendants
484 in their natural environment, suggesting evolution of local adaptation. In *C. vulgare* and
485 *L. hispidus*, we found evolution of faster germination. In contrast, we found less growth in terms
486 of plant size in the field conditions of the descendants from *C. vulgare* and lower production of
487 leaves of descendants from *L. hispidus* compared to their ancestors. This indicates evolutionary
488 differences, which did, however, not lead to lower mortality during that year's extreme drought
489 period. Furthermore, mortality, a clear indicator of selection pressure, was highest in summer,
490 where temperatures and drought peaked. These findings further support the notion that drought

491 has been a key selective force for evolution in recent years and that adaptations to prolonged
492 drought periods will play an important role for persistence of plant populations in the future
493 (Franks et al., 2007; Nevo et al., 2012; Sekor & Franks, 2018; Thomann et al., 2015; Vigouroux
494 et al., 2011). Evolutionary adaptations to drought include escape, avoidance and tolerance
495 strategies (Basu et al., 2016), but also the evolution of seed traits such as dormancy and faster
496 germination can be beneficial (Volaire and Norton, 2006).

497 Our study demonstrates that the incorporation of *in situ* transplantations into the resurrection
498 approach is an important step to infer evolutionary adaptation. In future resurrection studies,
499 the combination with *in situ* transplantations will be needed to confirm whether observed
500 strategy shifts in common garden experiments translate to improved plant performance in their
501 natural environments.

502 **6. Appendix**

503 **6.1. Author contribution**

504 PK, SG and JFS designed the study. SG and RR provided the seed material. PK, SG and JFS
505 conducted the experiment. PK, MMS and JFS analyzed the data. PK wrote the manuscript with
506 input from all co-authors.

507 **6.2. Acknowledgements**

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509 collection permits and for having authorized the experimental planting of *M. ciliata*. We thank
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511 and *C. vulgare*. We are also grateful to Louis-Marie Delescaille, Alain Bouchat and Jean-Louis
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518 for analyzing the soil samples. Finally, we thank the Royal Meteorological Institutes of Belgium
519 and the Netherlands for providing the climate data.

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523 **6.4. Data archiving**

524 The data associated with this manuscript will be uploaded to a repository upon acceptance.

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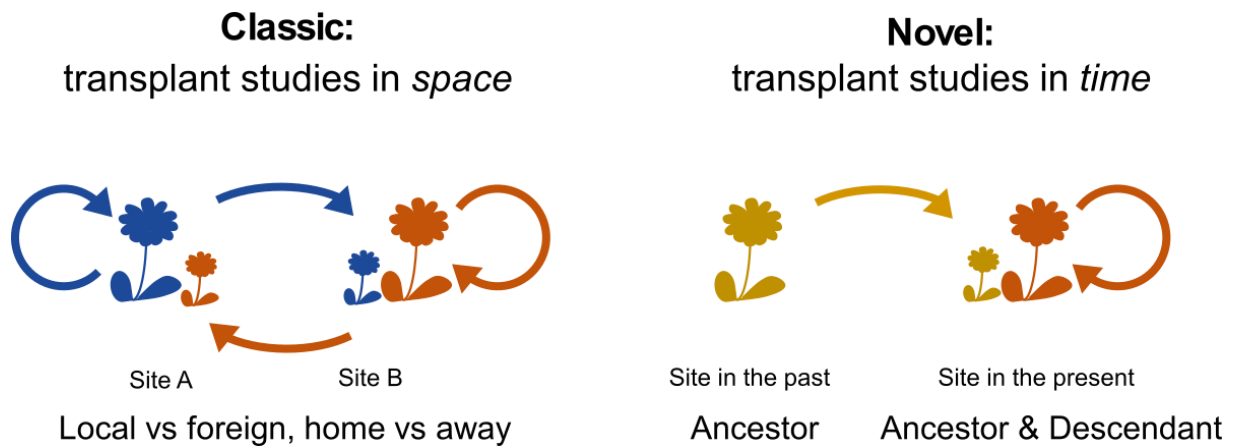
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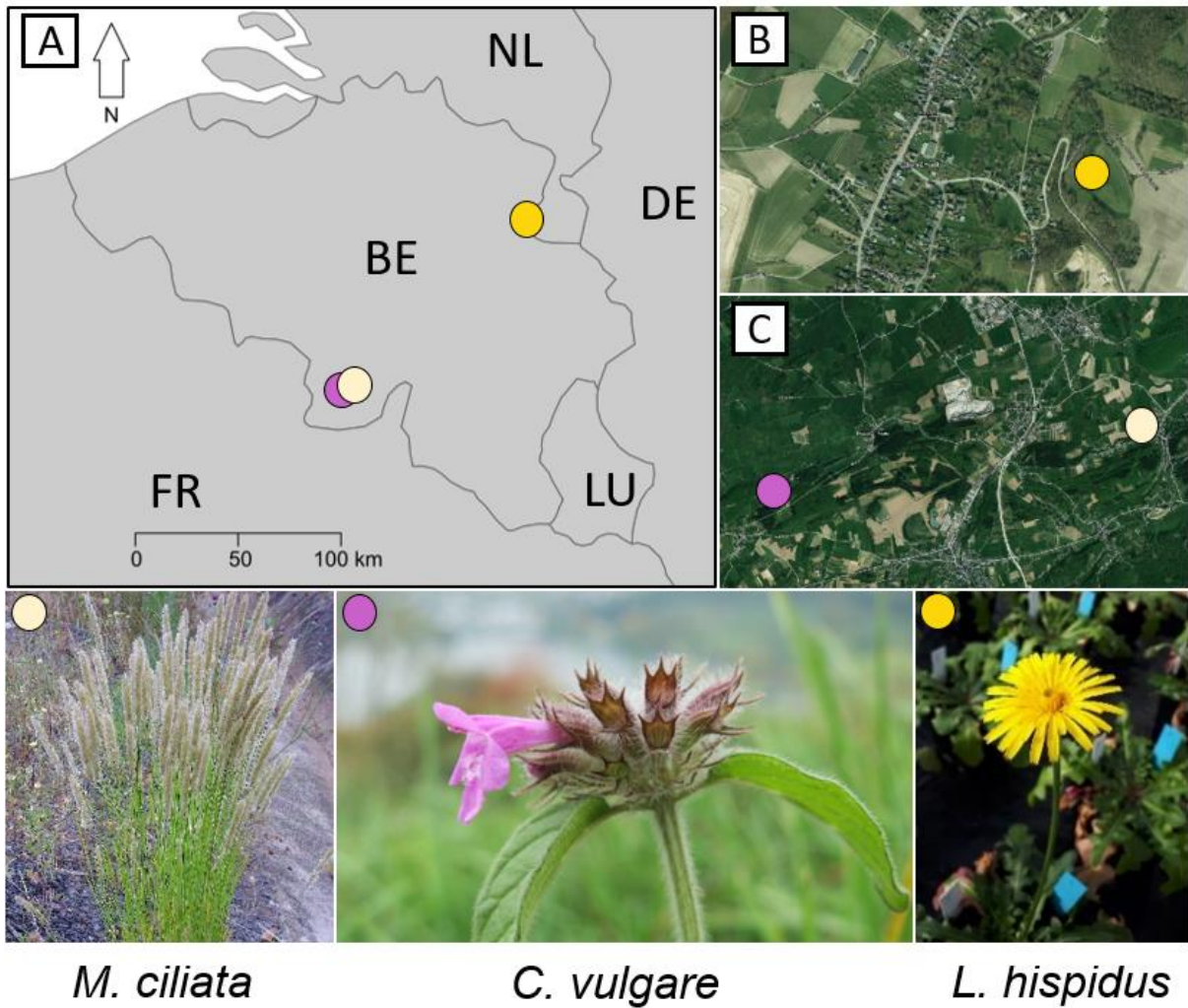
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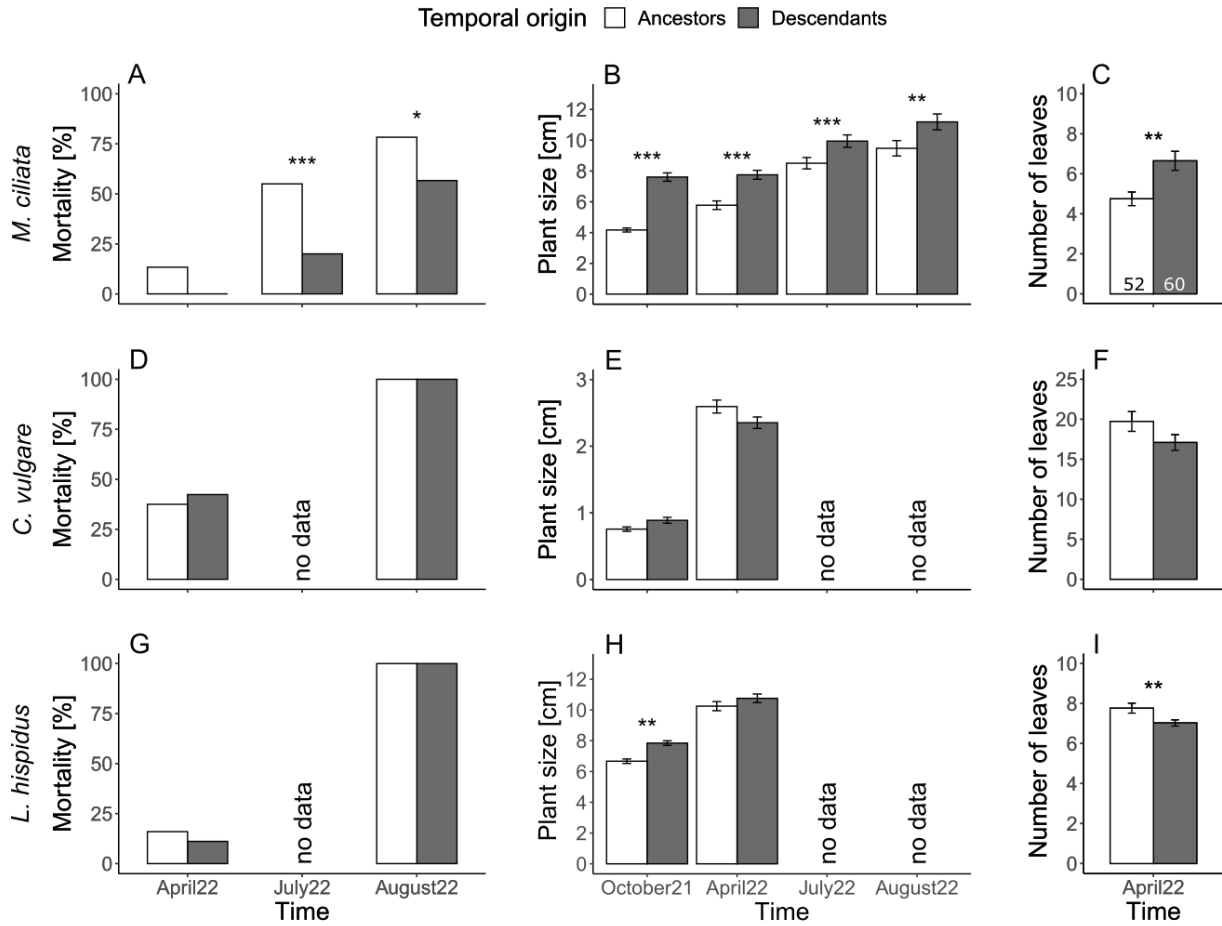
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677 **Figure 1.** Comparison of the classical approach of transplant experiments in space (left) with the novel approach
678 of transplant experiments in time (right). Classical reciprocal transplantation experiments aim to measure local
679 adaptation and fitness trade-offs by transplanting plants from different populations reciprocally to all respective
680 sites of origin. This method can conceptually be combined with the resurrection approach by transplanting
681 ancestors and descendants of the same population to their collection site today. By doing so, comparisons of
682 ancestors and descendants in their present-day natural environment can give insights into the evolution of local
683 adaptation and underlying processes through time.



684

685 **Figure 2.** Map of the focal populations in nature reserves in Belgium. Overview map of Belgium (A) and zoomed-
 686 in areas of the focal populations (B & C). The populations of the species *M. ciliata* (grey circle), *C. vulgare* (pink
 687 circle) and *L. hispidus* (yellow circle) are located in Belgian nature reserves. Map A was made in R (version 4.0.3,
 688 R Development Core Team 2022) using the packages *map* and *mapdata*. Maps B and C were made in QGIS (QGIS
 689 Development Team (2023) using the plugin *maptiler*. The photos of *M. ciliata* and *C. vulgare* are in the public
 690 domain.



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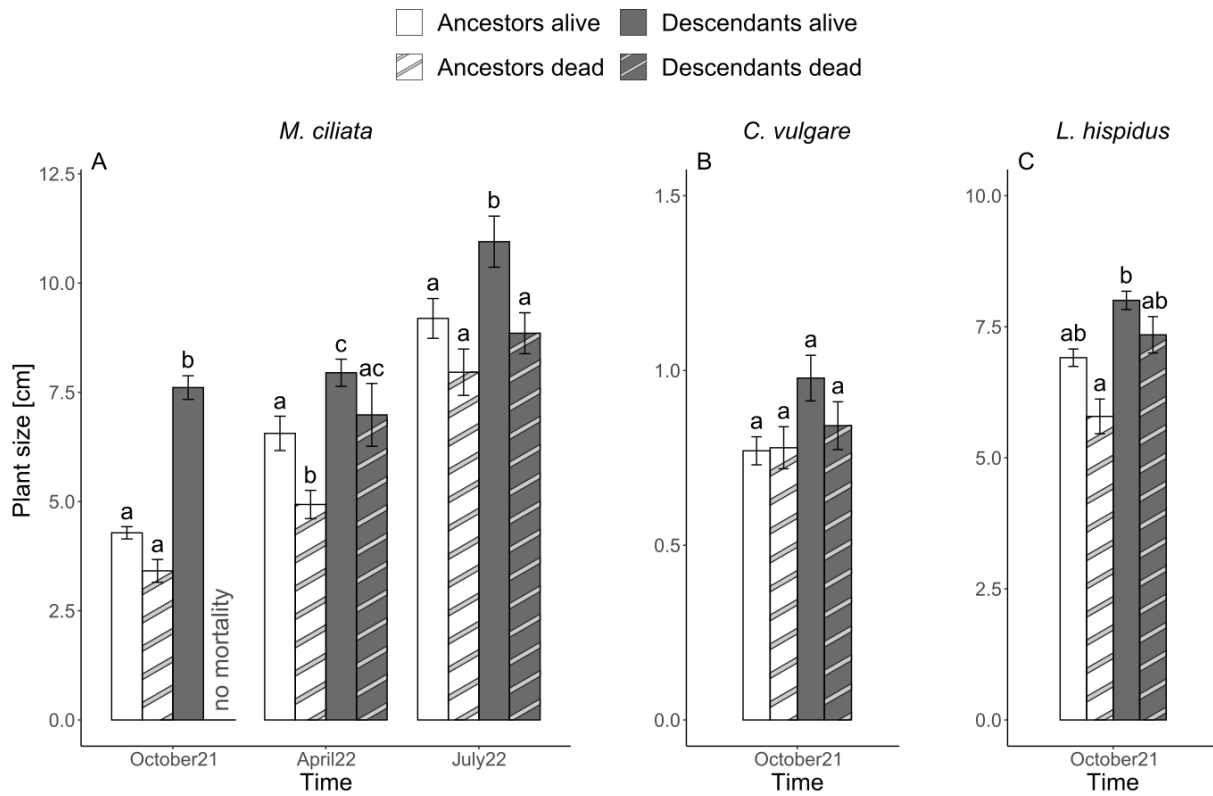
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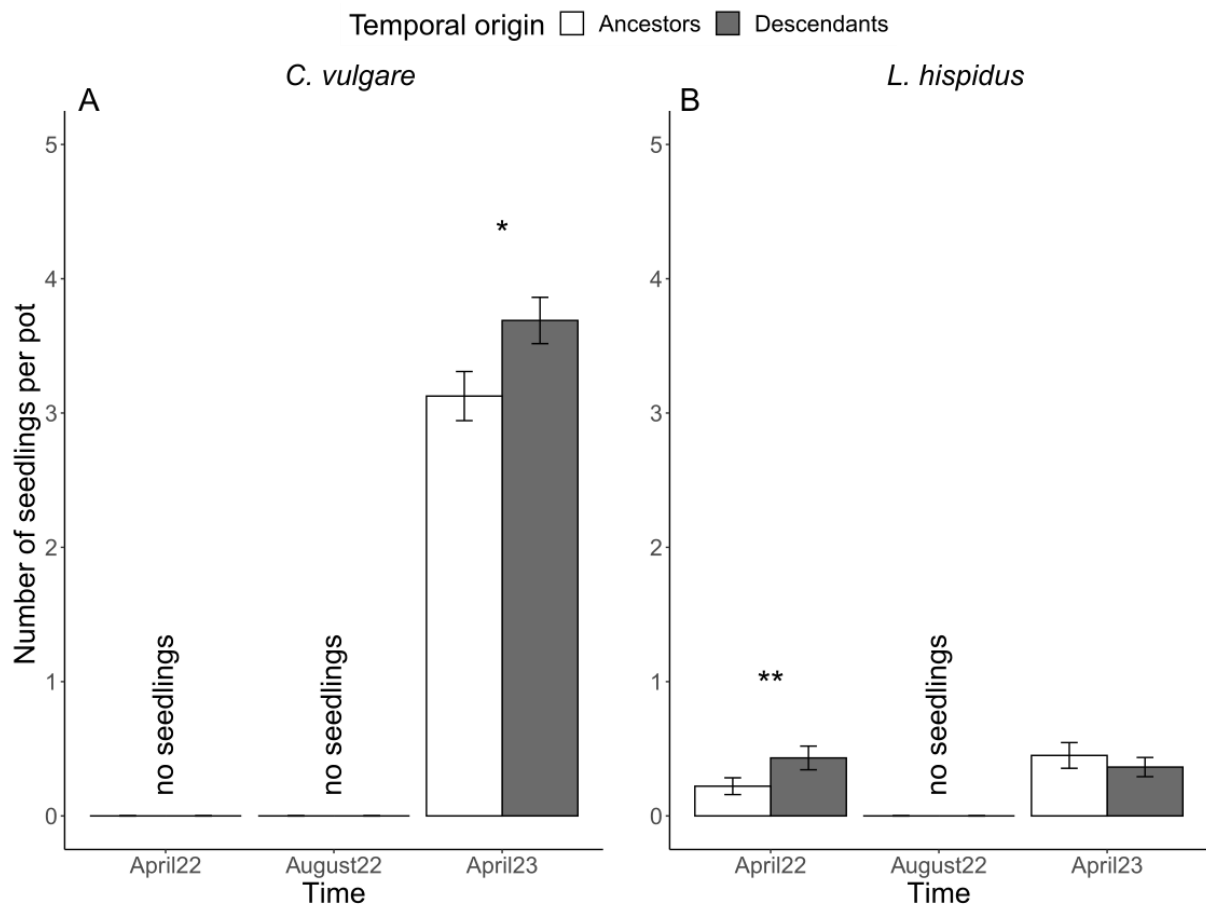
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Figure 3. Mortality rates, plant size and number of leaves of *Melica ciliata* (A, B, C), *Clinopodium vulgare* (D, E, F) and *Leontodon hispidus* (G, H, I) in ancestors (white bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors. Significant differences between ancestors and descendants in each point in time are indicated with asterisks ($p = 0.05 - 0.01$ *; $p = 0.01 - 0.001$ **; $p < 0.001$ ***). The sample sizes are indicated in the bars.



698

699 **Figure 4.** Plant size of *Melica ciliata* (A), *Clinopodium vulgare* (B) and *Leontodon hispidus* (C) grouped by their
 700 mortality status at the next measurement (dead indicated with stripes and alive indicated without stripes) of
 701 ancestors (white bars) and descendants (grey bars) at three different time points. Shown are means and standard
 702 errors. Significant differences in plant size between groups are shown with different letters within each time of
 703 measurement ($p < 0.05$). Note that we recorded 100 % mortality in *L. hispidus* and *C. vulgare* in August 2022,
 704 hence only the time period October 2021 – April 2022 could be analyzed.



705

706 **Figure 5.** Number of seedlings germinated per pot from *C. vulgare* (A) and *L. hispidus* (B) in ancestors (white
 707 bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors.
 708 Significant differences between ancestors and descendants are indicated with asterisks ($p = 0.05 - 0.01$ *; $p = 0.01$
 709 $- 0.001$ **).

710 **Table 1.** Results of the statistical models testing the effects of temporal origin (ancestors, descendants), time of
711 measurement (if measured more than once) and their interaction on mortality (GLMM), plant size (LMM) and
712 number of leaves (LMM) of *Melica ciliata*, *Clinopodium vulgare* and *Leontodon hispidus*. For plant size, an
713 additional LMM was created with temporal origin, time of measurement, mortality and their two- and three-way
714 interactions. Shown are F-values and p-values for LMMs and Chi² and p-values for GLMMs. Significant p-values
715 (< 0.05) are shown in bold.

Response variable	Explanatory variable	<i>M. ciliata</i>		<i>C. vulgare</i>		<i>L. hispidus</i>	
		Chi ²	p	Chi ²	p	Chi ²	p
Survival	Origin	20.73	<0.001	0.91	0.339	1.00	0.316
	Time	58.90	<0.001				
	Origin × Time	0.99	0.804				
		F	p	F	p	F	p
Plant size	Origin	51.36	<0.001	0.05	0.820	7.32	0.013
	Time	82.31	<0.001	706.17	<0.001	272.03	<0.001
	Origin × Time	6.63	<0.001	7.83	0.006	4.47	0.035
Leaf number	Origin	9.63	0.002	1.86	0.185	11.80	0.002
	Origin	45.13	<0.001	2.29	0.144	8.91	0.005
	Time	65.23	<0.001				
	Survival	13.29	<0.001	2.86	0.092	3.63	0.058
Plant size	Origin × Time	6.36	0.002				
	Origin × Survival	0.01	0.919	0.87	0.351	0.71	0.399
	Time × Survival	0.01	0.986				
	Origin × Time × Survival	3.24	0.073				

716

717

718 **Table 2.** Results of the statistical models testing the effects of temporal origin (ancestors, descendants), time and
719 their interaction on number of seedlings after sowing (GLMM) of *Clinopodium vulgare* and *Leontodon hispidus*.
720 Shown are Chi² and p-values. Significant p-values (< 0.05) are shown in bold. For *C. vulgare*, we only recorded
721 seedlings at one time point (April 2023), hence we tested only the effect of temporal origin.

Response variable	Explanatory variable	<i>C. vulgare</i>		<i>L. hispidus</i>	
		Chi ²	p	Chi ²	p
Number of seedlings	Origin	4.719	0.030	1.103	0.294
	Time			1.935	0.380
	Origin × Time			8.666	0.013

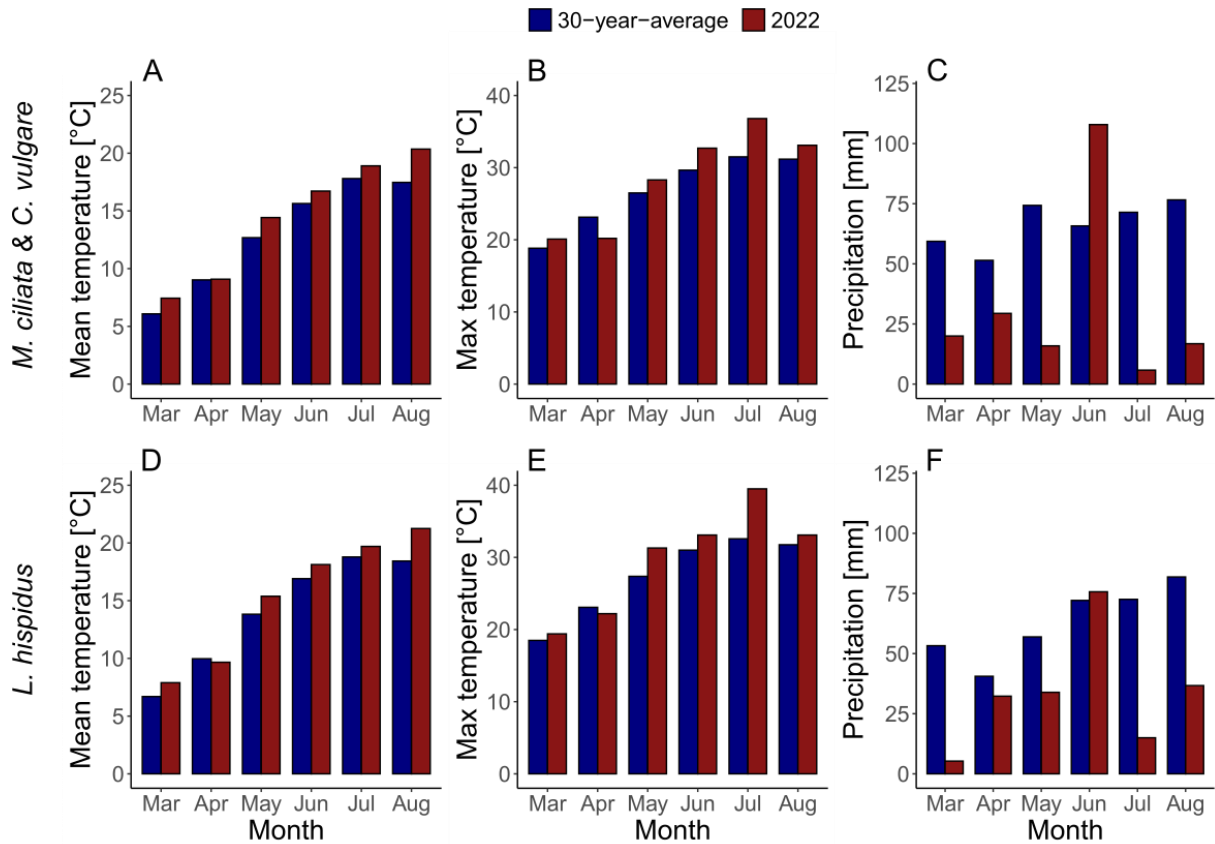
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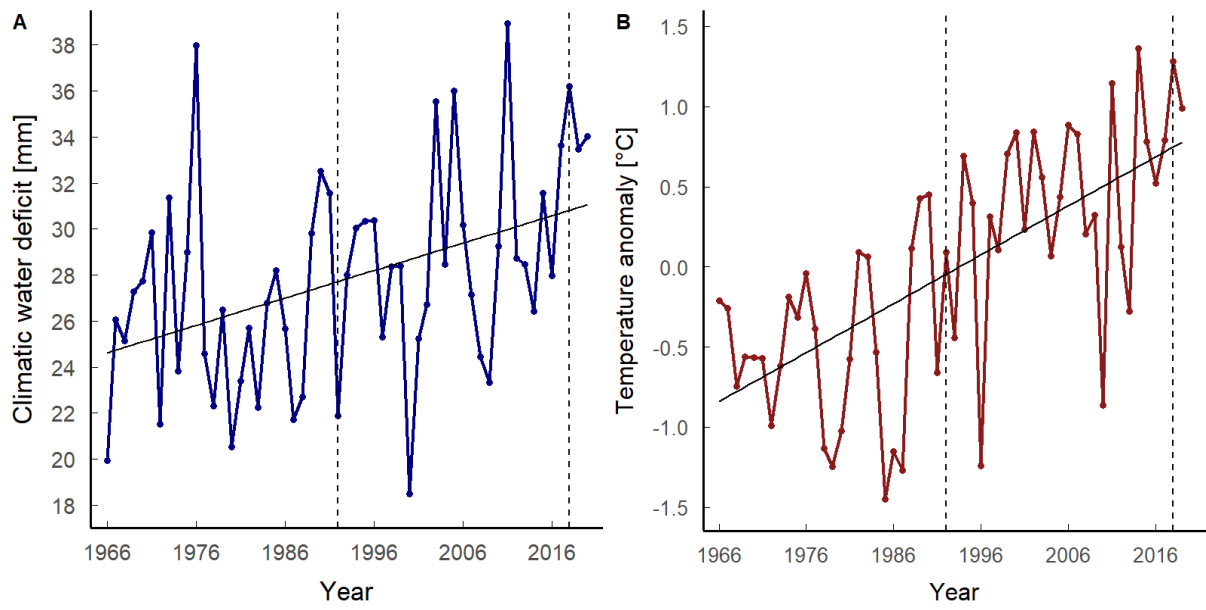
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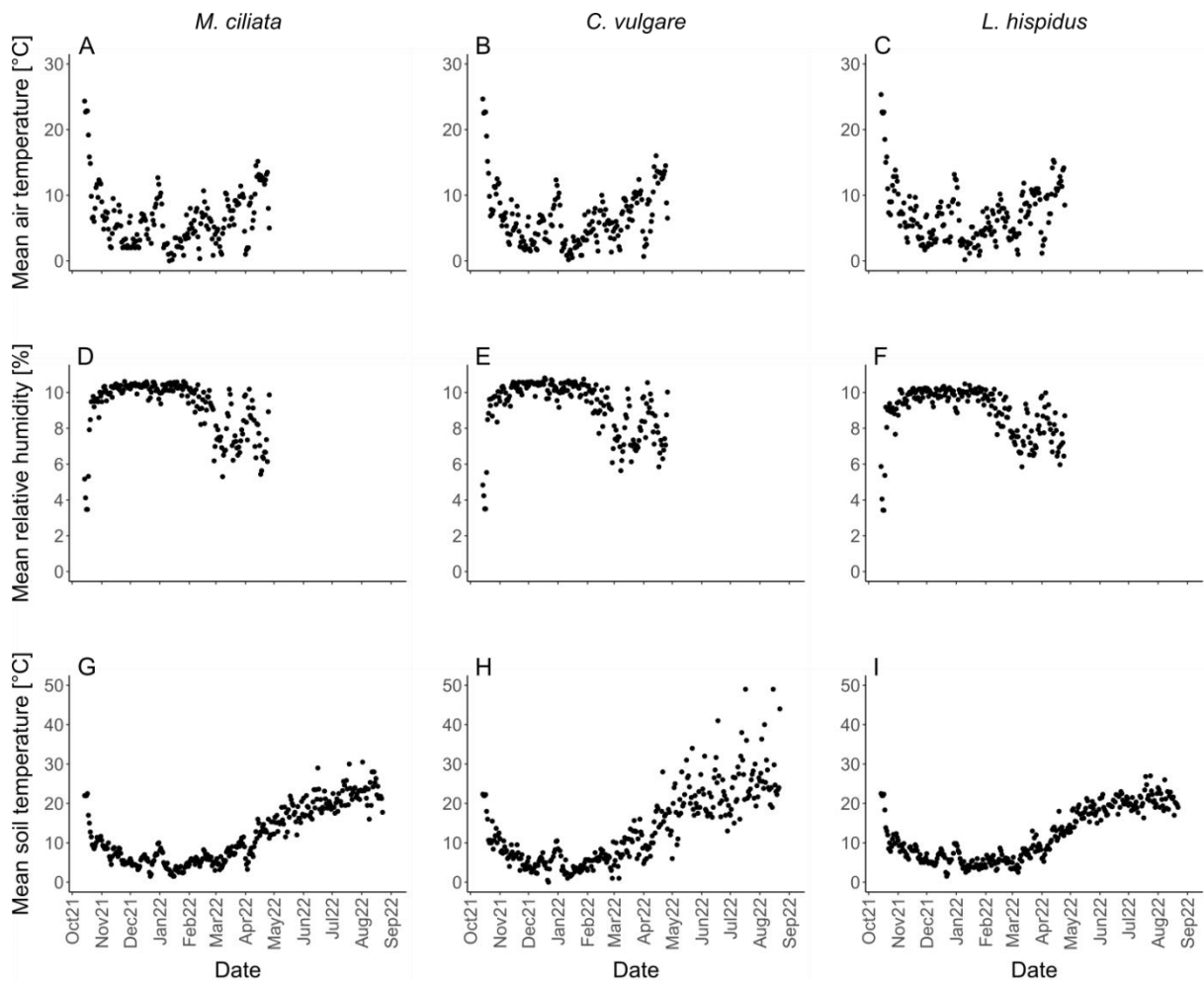


729 **Figure S1.** Climate data from a weather station near *Melica ciliata* and *Clinopodium vulgare* (A, B & C) and a
 730 weather station near *Leontodon hispidus* (D, E & F) showing mean temperature [°C], max temperature [°C] and
 731 precipitation [mm] during the months May – August. The average of 1989 – 2019 (30-year average) is shown in
 732 blue bars and the year 2022 is shown in red bars. The weather station of Dourbes is located 3 km from the *M.*
 733 *ciliata* and 14 km from the *C. vulgare* population. The weather station of Maastricht is located 14 km from the *L.*
 734 *hispidus* population.
 735



736

737 **Figure S2.** Temperature anomaly (A) and climatic water deficit (B) over the last four decades of *Melica ciliata*
 738 and *Clinopodium vulgare* populations. The dotted lines refer to the seed collection years (ancestors 1992 vs.
 739 descendants 2018).



741

742 **Figure S3.** Daily mean values of air temperature [°C] relative humidity [%] and soil temperatures [°C] for *Melica*
 743 *ciliata* (A, D, G), *Clinopodium vulgare* (B, E, H) and *Leontodon hispidus* (C, F, I) during the experimental period
 744 from October 2021 to September 2022. Air temperature and relative humidity was measured by a shaded data
 745 logger (iButton DS1923, Maxim Integrated) in the center of each plot. Mean soil temperature was measured by
 746 two data loggers (iButton DS1921G-F5, Maxim Integrated) buried 5 cm deep into the soil in two opposing corners
 747 of each experimental plot. All data loggers recorded one value every 4 hours. Data for relative humidity and air
 748 temperature only extends until April 2022, because the loggers malfunctioned afterwards.

749 **Table S1.** Coordinates, collection years and number of seed families of the study species.

Species	Coordinates	Collection years	Number of seed families in 2018/2020	Estimated population size in 2018
<i>Clinopodium vulgare</i>	50°03'55"N 4°26'40"E	1992/2018	47	500
<i>Melica ciliata</i>	50° 4' 34" N 4°32'35"E	1992/2020	21	25
<i>Leontodon hispidus</i>	50°47'35"N 5°40'25"E	1995/2018	20	100

750

751

752 **Table S2.** Results of the statistical models for *Melica ciliata* testing the effects of the dataset (subset vs. complete)
 753 on mortality, plant size and number of leaves. The model for the number of leaves (LMM) contained no other
 754 explanatory variables, whereas the model for plant size (LMM) and for mortality (GLMM) included the time of
 755 measurement and the dataset \times time interaction. Shown are F-values and p-values for the LM and LMM and Chi²-
 756 and p-values for the GLMM. Significant p-values (< 0.05) are shown in bold.

Response variable	Explanatory variable	<i>M. ciliata</i>	
		Chi ²	p
Survival	Dataset	0.01	0.935
	Time	21.55	< 0.001
	Dataset \times Time	54.98	< 0.001
Plant size		F	p
	Dataset	0.23	0.630
	Time	85.46	< 0.001
	Dataset \times Time	0.03	0.994
Ramets	Dataset	0.01	0.915
	Time	32.51	< 0.001
	Dataset \times Time	0.07	0.934

757

758

759 **Table S3.** Results of the statistical models testing the effects of time of measurement (only *M. ciliata* due to
 760 multiple measurements), plant size and their interaction on mortality (GLMM) of *Melica ciliata*, *Clinopodium*
 761 *vulgare* and *Leontodon hispidus*. Shown are F-values and p-values. Significant p-values (< 0.05) are shown in
 762 bold.

Response variable	Explanatory variable	<i>M. ciliata</i>		<i>C. vulgare</i>		<i>L. hispidus</i>	
		Chi ²	p	Chi ²	p	Chi ²	p
Mortality	Time	31.256	< 0.001				
	Plant size	22.334	< 0.001	1.594	0.207	9.481	0.002
	Time \times Plant size	3.515	0.172				

763

764 **Table S4:** Chemical composition of soil samples taken at the collection sites and the transplantation sites of our
 765 study species *Melica ciliata*, *Clinopodium vulgare* and *Leontodon hispidus*. We took four samples of 25 cm² soil
 766 each at 10 cm depth at random positions in each site. The four samples from each location were mixed together
 767 and analyzed to determine the amount of fundamental minerals for plant development (i.e., plant-available P, K,
 768 S, Ca and Mg, total element content of P, K, S, Ca, total C, N and S), as well as pH level and salinity.

Species	Site	pH	Salinity [μ S/cm]	N [%]	C [%]	TIC [%]	C org [%]	C/N	S [%]
<i>M. ciliata</i>	Collection	7.45	308	1.28	15.47	1.02	14.45	11.26	0.04
	Transplantation	7.13	251	0.95	12.36	0.16	12.2	12.81	0.04
<i>C. vulgare</i>	Collection	6.56	114	0.53	6.45	0.01	6.44	12.17	0.02
	Transplantation	6.62	88	0.43	4.6	0.04	4.56	10.67	0.02
<i>L. hispidus</i>	Collection	6.46	88	0.49	6.9	0	6.9	14.14	0.01
	Transplantation	6.52	127	0.76	11.01	0.01	11	14.51	0.03

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