



# Surviving the extinction vortex? Discovering remnant stands of *Senecio hercynicus* (Compositae, Senecioneae) evading genetic swamping by its congener *S. ovatus* in the Bavarian and Bohemian Forest region

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## ABSTRACT

Genetic swamping by introgressive hybridisation threatens diversity, caused by climate warming particularly in mountainous regions worldwide. Recent studies resulted in a threatening perspective for *Senecio hercynicus* in the Bavarian Forest due to genetic swamping by introgressive hybridisation with its congener *S. ovatus*. To examine the situation more closely, the distribution and hybridization of *S. hercynicus* and *S. ovatus* in high elevation regions of the Bavarian and Bohemian Forest was analyzed by restriction enzyme digestion of nrDNA ITS1 (PCR-RFLP; PCR restriction fragment length polymorphism). For a total of 706 samples from 94 different sample localities a hybrid index was inferred from the fragment band intensities after PCR-RFLP digestion. Boxplot diagrams of the hybrid indices show a tendency of western populations towards *S. ovatus* genotypes and for populations to the east towards *S. hercynicus* genotypes. When the hybrid index data was subjected to a regression analysis with nine factors (five habitat patterns inferred during sampling and four bioclimatic variables), only geographical longitude and latitude seemed to describe the observed distribution of *S. hercynicus* and *S. ovatus* significantly, arguing for the distribution and hybridisation patterns being shaped rather due to historical than to eco-climatological determinants. While a broad zone of hybridisation between the two species in the Bavarian and Bohemian Forest region was inferred, our study demonstrates that purebred *S. hercynicus* still exists and remnant stands of this species should be the target of species conservation measures.

## 1. Introduction

Besides habitat loss and habitat change caused by radical land-use changes, rapid climate change, or the introduction of invasive species, hybridisation between formerly isolated, but closely related species is also considered a threat to plant species diversity (Rhymer and Simberloff 1996; Todesco et al., 2016; Ottenburghs 2021; Sher 2022). This so-called ‘genetic swamping’ may be especially pronounced in mountainous habitats, where climate-induced range shifts of plant species will promote genetic contact of formerly allopatric species (Grabherr et al. 1994; Lenoir et al. 2008; Truong et al. 2007). Additionally, a shifting plant phenology in response to global change may further promote gene flow between closely-related, sympatric species with formerly effective temporal isolation barriers (Cleland et al. 2007; Parmesan 2006; Sherry

et al. 2007). Due to their overlapping geographical and elevational distribution ranges, the formation of mixed stands caused by their similar habitat preferences, and their reproductive isolation secured by differences in flowering time, the *Senecio nemorensis*-group (Compositae, Senecioneae) is a suitable plant group for the study of genetic swamping caused by environmental changes.

In a row of contributions during the last two decades, we have documented and studied the genetic swamping of *Senecio hercynicus* Herborg by its congener *S. ovatus* (G.Gaertn. et al.) Hoppe in the Bavarian and Bohemian Mountain ranges in SE Germany (Oberprieler et al. 2010, 2015, 2016; Bog et al. 2017a). While the latter species is common and widely distributed throughout central, eastern, and south-eastern Europe, the former shows a disjunct distribution area limited to higher elevations of the low mountain ranges of central

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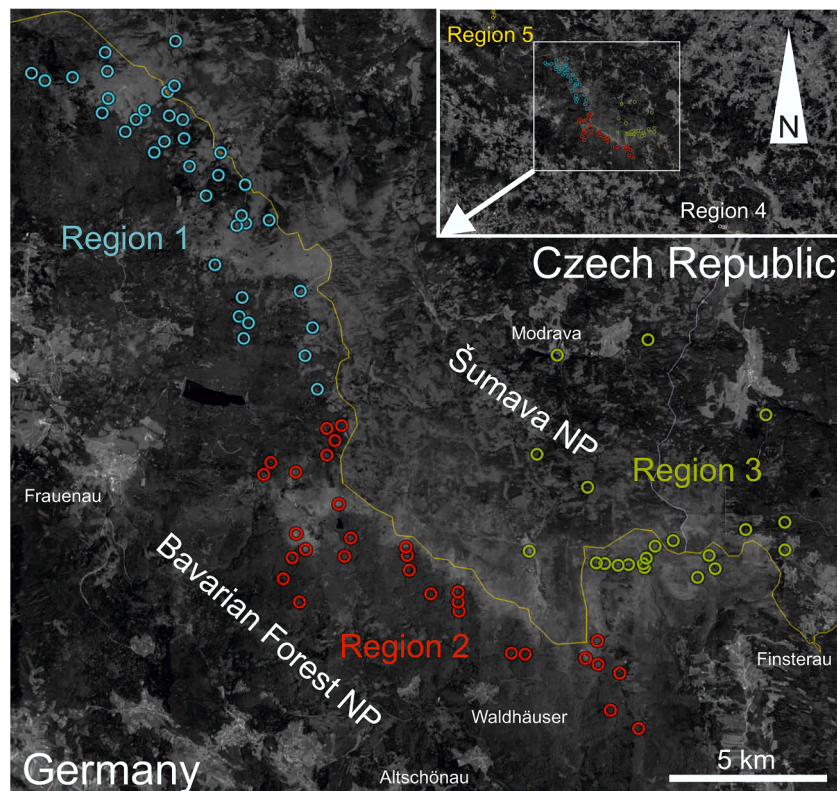
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**Fig. 1.** Geographical positions of the 94 *Senecio* stands sampled for the present survey. While regions 1–3 are located in the Bavarian Forest National Park and adjacent areas of the Šumava National Park in the Czech Republic, regions 4 and 5 (insert) are located in the NW and the SE of the national park regions along the Bavarian-Bohemian Forest ridge (map based on Google Earth Pro v.7.3.6.9796).

Europe, parts of the Alps, and the mountains of the Balkan Peninsula. Due to their overlapping geographical and elevational ranges and their shared habitat preferences (forest clearings, forest roadsides, forest edges, and sparse high-elevation spruce forests) mixed stands are often encountered. In some of these, flowering time is so different – with *S. herbicynicus* flowering around 3–4 weeks before *S. ovatus* – that hybridisation is not observed. This phenology-based reproductive isolation has also been demonstrated by common-garden experiments (Herborg 1987; Bog et al. 2017b). However, caused by the intermediate flowering time of occasionally formed, fully fertile F1 hybrids and the lack of any other reproductive barrier, hybrid swarms of these two, morphologically distinct species occur quite often.

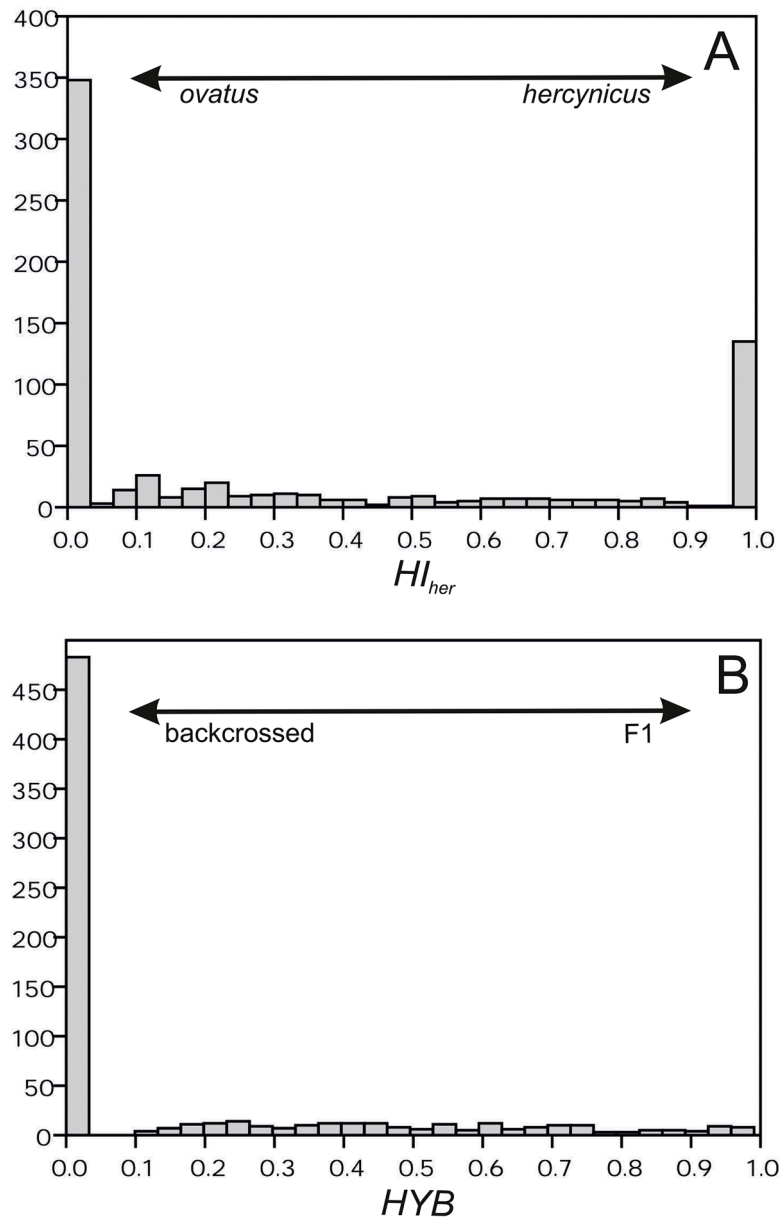
Corroborating morphological evidence provided by Herborg (1987) and Oberprieler (1994), the introgressive hybridisation between these two members of the *Senecio nemorensis* syngameon has been confirmed using molecular techniques by Raudnitschka et al. (2007; RAPD fingerprinting) and Oberprieler et al. (2010; AFLP fingerprinting). More detailed studies along elevational gradients in the Bavarian Forest National Park (Oberprieler et al. 2015; Bog et al. 2017a) subsequently demonstrated that (a) genetic swamping of *S. herbicynicus* by *S. ovatus* has already reached the highest habitats of the former species at around 1300 m a.s.l., where the presence of purebred *S. herbicynicus* individuals were documented through herbarium specimens (Herborg 1987; Oberprieler 1994), and (b) climatologically mediated divergent selection regimes along elevational gradients are not sufficiently strong to re-establish genetically isolated species.

Owing to the strict limitation of sampling along four elevational gradients in the Bavarian Forest National Park for these former studies, large parts of the high-elevation zones of the Bavarian-Bohemian Forest Mountain ridge were left unsurveyed. Personal observations by the first author of the present contribution during the early 90s of the last century, however, suggested that populations of purebred *S. herbicynicus*

might still exist off these transects. As a consequence, we decided to enlarge the sampling of *Senecio* stands to the high-elevation zones of the whole Bavarian Forest National Park (both in its older parts between Mt Rachel and Mt Lusen, termed region 2 in the present study, and the more recently added parts between Mt Rachel and Mt Falkenstein, region 1) and adjacent parts of the Šumava National Park across the border with the Czech Republic. Additional stands were sampled at other high-elevation regions of the ridge; i.e., region 4 around Mt Dreisessel and Mt Plöckenstein (SE of the two national parks) and region 5 around Mt Osser (NW of the national parks). By using a fast genotyping procedure based on a sequence difference between the two species at the nuclear-ribosomal (nrDNA) internal transcribed spacer 1 (ITS1) region, we were able to include more than 700 plant individuals from 94 different stands into our present analysis. Using this multi-copy marker also as a fine-scaled measure of the genetic constitution of each individual and its position on a hybridisation gradient between the two parental genotypes enabled us additionally to infer correlations of hybridisation patterns with eco-climatological and other habitat descriptors. Finally, eco-climatological niche modelling was done based on information from revised herbarium specimens throughout the distribution ranges of the two species in order to infer to what extent the ecological niches of *S. herbicynicus* and *S. ovatus* are different.

## 2. Materials and methods

**Sampling.** – Plant material (721 individuals) was collected between May 22, 2017 and July 20, 2017 (see Table S1 in the Supplementary Material for details on geographical positions and information on habitat and eco-climatological conditions of the 94 plots). As seen in Fig. 1, only high elevation areas of the Inner Bavarian and Bohemian Forest were chosen for sampling, ranging from 933 m a.s.l. up to 1345 m a.s.l. Sampled populations were coarsely grouped into five different



**Fig. 2.** Results of the nrDNA ITS1 fingerprinting in 94 *Senecio* stands along the Bavarian-Bohemian Forest ridge. Summarising histograms of absolute frequencies for the hybridisation index ( $HI_{her}$ ) and the hybridity index ( $HYB$ ) observed in all surveyed 721 plants.

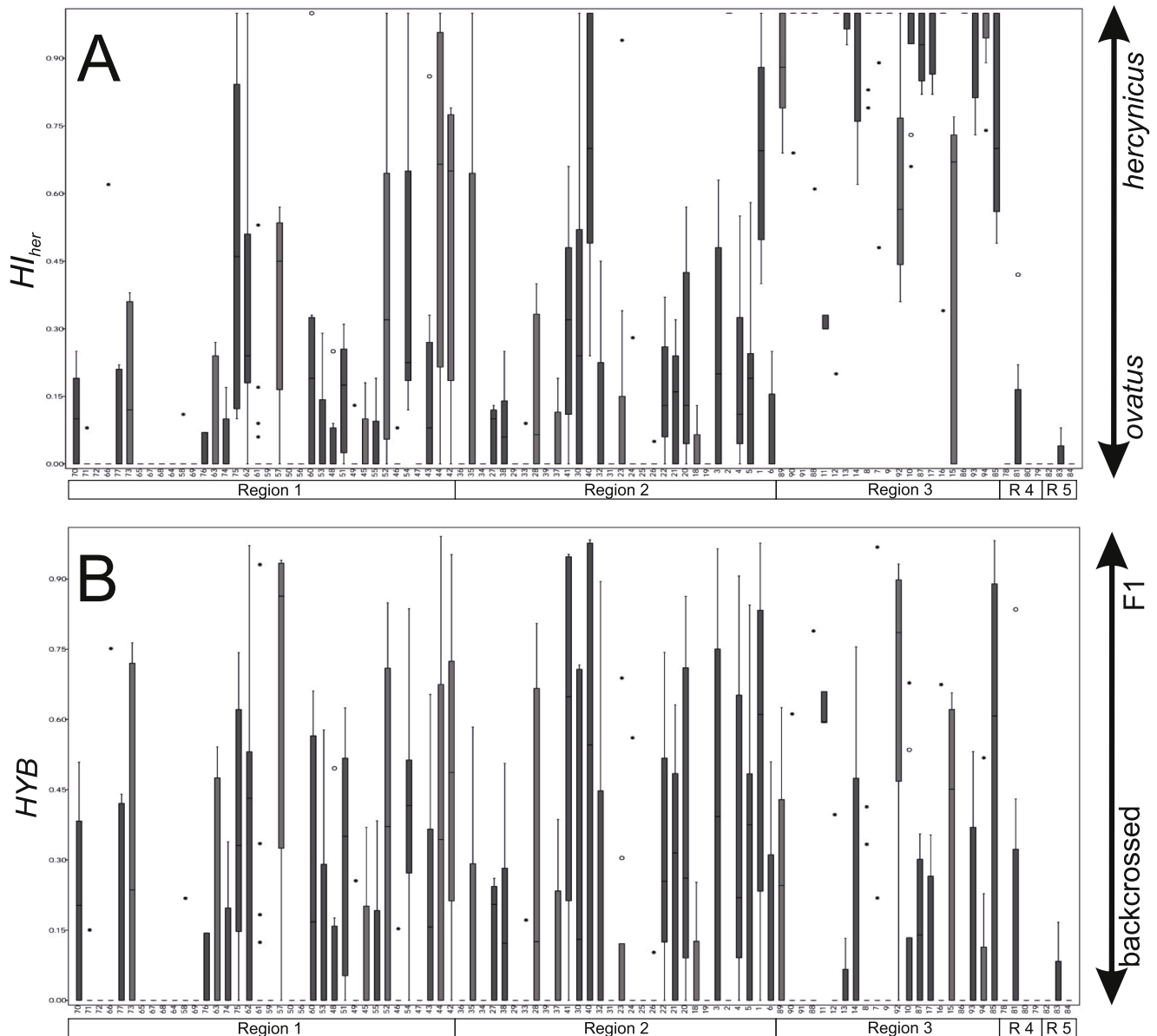
regions: while region 1 represents the so-called expansion area of the Bavarian Forest National Park together with some individuals from the adjacent parts of the Šumava National Park, region 2 consists of sample localities in the older parts of the Bavarian Forest National Park (the Rachel-Lusen area), and region 3 plots come from the very eastern part of the Bavarian Forest NP and adjacent parts of the southern Šumava NP across the border with Czech Republic. Finally, region 4 is the zone around Mt Dreisessel and Mt Plöckenstein (four populations) and region 5 is restricted to the Mt Osser region (three populations).

The plant material was taken on plots selected for being as representative as possible to cover the potential distribution range of *S. hercynicus* in the region, taking into account sample sizes, distance to the neighbouring plots, and geographical location. If plants grew in clonal aggregations, only one sample per clone was taken. This led to different sample sizes per plot, which ranged between 2 and 21 individuals sampled. GPS data for sampled populations (*latitude*, *longitude*, *elevation*) were taken by 'Locus Map' (Asamm Software s.r.o. 2017) and additional habitat information was recorded for each site (*creek*:

adjacency to a creek coded 0/1; *road*: adjacency to a forest road coded 0/1). Sampled individuals were selected without accounting for their morphological and phenological appearance and taxonomic affiliation. From each chosen plant, a single leaf was sampled and stored in a paper bag (tea filter), which was immediately cooled in the field and frozen at  $-18^{\circ}\text{C}$  after returning from the collection trips.

**nrDNA ITS1 PCR-RFLP analysis.** – Genotyping individuals took advantage of a sequence difference in the internal transcribed spacer region 1 of the nuclear ribosomal repeat (nrDNA ITS1) at positions ITS1-90/91 (Bog et al., 2017b; 5'-GGACACC-3' in *S. ovatus* vs. 5'-GGATGCC-3' in *S. hercynicus*) and the restriction enzyme *BseGI* with its motive of 5'-GGATGNN-3': on the one hand, genotype 1 (*S. ovatus*) is not digested and the 500 bp long PCR product of nrDNA ITS1 remains intact, on the other, genotype 2 (*S. hercynicus*) is digested at position ITS1-90/91, which leads to two fragments of about 300 bp and 200 bp length. Consequently, a hybrid individual causes a mixture of all three fragments (500 bp, 300 bp, 200 bp).

For amplifying the nrDNA ITS1 region, a direct PCR method without



**Fig. 3.** Results of the nrDNA ITS1 fingerprinting in 94 *Senecio* stands along the Bavarian-Bohemian Forest ridge. (A) Box and whisker plot summarising population-wise values for the hybridisation index ( $HI_{her}$ ) in the five sampled regions (arranged according to geographical longitude within regions). (B) Box and whisker plot summarising populations-wise values for the hybridity index ( $HYB$ ).

any prior DNA extraction procedure was performed following a modified scheme described by Bog et al. (2017b). A master mix of 11  $\mu$ l was prepared containing 4.25  $\mu$ l H<sub>2</sub>O, 6.25  $\mu$ l of a twofold concentrated Master Mix RED with 1.5 mM MgCl<sub>2</sub> (Ampliqon, Odense, Denmark), and 0.25  $\mu$ l of each primer [ITS-18SF: 5'-GAA CCT TAT CGT TTA GAG GAA GG-3' (Rydin et al., 2004) and ITS1-P2: 5'-CTC GAT GGA ACA CGG GAT TCT GC - 3' (Ochsmann, 2000)]. Finally, a sample leaf-tissue disc was punched out of a thawed leaf with a 200  $\mu$ l pipette tip (Sarstedt, Nümbrecht, Germany) and was added to the mixture. The PCR was performed at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, another 15 s at 55 °C and 30 s at 72 °C, and a final extension cycle for 3 min at 72 °C. Subsequently, PCR products were checked quantitatively and qualitatively by applying 2.5  $\mu$ l of the product to a gel electrophoresis with a 1.5 % agarose gel in 1x TAE-buffer and 120 V for 40 min. If the PCR had failed or produced too small quantities of the amplicon, the PCR scheme was changed for the sample concerned in the following manner: instead of putting the sample disc directly into the PCR mixture,

it was mixed with 20  $\mu$ l of water, from which then 1.5  $\mu$ l were pipetted to the PCR master mix of 11  $\mu$ l (see above). The temperature settings stayed the same, except of lowering the annealing temperature from 55 °C to 45 °C.

To digest the resulting PCR product (about 500 bp in length), a modified method described by Bog et al. (2017b) was used. Six  $\mu$ l of the amplicon mixture were added to 4  $\mu$ l of a digestion mix, which consisted of 2.5  $\mu$ l H<sub>2</sub>O, 1  $\mu$ l Buffer Tango (FisherScientific, Schwerte, Germany) and 0.5  $\mu$ l of the restriction enzyme *BseGI* (FisherScientific, Schwerte, Germany). Subsequently, the mixture was kept at 55 °C for 8 h, followed by 80 °C for 20 min for enzyme inactivation. Finally, the digestion product was applied to a gel electrophoresis with a 2 % agarose gel in 1x TAE-buffer and 120 V for 40 min and a digital photograph of the gel was taken.

For making statements about the hybridisation state of an individual, the gel image was analyzed with GelAnalyzer2010 (Lazar Software, developed by Dr. Istvan Lazar, Debrecen, Hungary). After lanes were set

**Table 1**  
Results of the fitted regression models.

	Estimate	Std.Error	df	T/Z value	P value
<b>(a) Generalised Linear Mixed-Effect Model (<math>HI_{her} = 0/1</math>)</b>					
Intercept	-6.742	2.156		-3.128	0.002**
scale(latitude)	15.323	5.696		2.690	0.007**
scale(longitude)	27.125	7.945		3.414	< 0.001***
<b>(b) Linear Mixed-Effects Model (<math>0 &lt; HI_{her} &lt; 1</math>)</b>					
Intercept	0.37553	0.02096	62.74	17.92	< 0.001***
scale(latitude)	0.12986	0.03648	66.81	3.56	< 0.001***
scale(longitude)	0.23341	0.03468	73.03	6.73	< 0.001***
<b>(c) Linear Mixed-Effects Model (<math>HYB &gt; 0</math>)</b>					
Intercept	0.48226	0.02114	61.10	22.81	< 0.001***
scale(longitude)	0.04642	0.02027	73.10	2.29	0.025*

manually, bands were detected automatically, with corrections added whenever there were disturbances in the gel or the program made an obvious mistake. Background subtraction used the ‘rolling ball’ method, for which the ‘ball radius’ was set to 25 units, to get intensities independent of the gel’s background signal. Subsequently, the raw volume below the band peaks was measured and filled into an Excel spreadsheet. Eventually, the intensities of all three bands concerned were summed up and taken as a denominator, whereas the sum of intensities of the two *S. hircynicus* bands was taken as a numerator. The result of this quotient describes the hybridization state of each individual plant and is called hybridisation index ( $HI_{her}$ ) in the following. Finally, the hybridity index ( $HYB$ ) describing the genetic ‘intermediarity’ of an individual was derived from the previously calculated  $HI_{her}$ .  $HYB$  describes the degree of hybridity exhibited by an individual, ranging from 0 for purebred individuals to 1.0 for individuals with a hybrid index of  $HI_{her} = 0.5$ .

**Data analysis.** – Due to the interval from 0 to 1 and the nature of hybridisation, the frequency of hybrid indices is very unequally distributed and therefore a beta distribution emerged (seen in Fig. 2). Since the statistical analysis of a beta distributed dependent variable was too demanding, the samples had to be split up in two separate analyses. Purebred individuals with  $HI_{her} = 0$  and 1 were subjected to a Generalized Linear Mixed-Effects Model (*glmer*), whereas hybrid genotypes with  $0 < HI_{her} < 1$  were modelled with a Linear Mixed-Effects Model (*lmer*). A total of nine factors (fixed effects) were tested for a model to best predict the  $HI_{her}$  and  $HYB$  values, five of them were inferred during sampling of plant material (*latitude*, *longitude*, *creek*, *road*, *elevation*) and four variables [annual mean temperature (*LT\_MW*), annual sum of rainfall (*NIED\_MW*), annual global radiation (*GR\_MW*), annual relative air humidity (*RF\_MW*)] were gained from a climate model (Klößing 2018). Also, a tenth factor (*population*) was added to represent the random effect in the model. Because the climate model does not include data for the Dreissessel area, plots 78–81 (region 4) had to be skipped in this analysis. Prior to the statistical evaluation, some of the variables had to be rescaled to a distribution with a mean of 0 and variance of 1. This was done by the command ‘scale()’ in R v.2022.07.2 (R Development Core team, 2009–2022).

The *glmer* for purebred individuals was fitted by model simplification also done with R v.2022.07.2 (R Development Core Team, 2009–2022). The *glmer* procedure was started with all factors included and the results were examined. Then non-significant (statistical significance:  $P < 0.05$ )

**Table 2**  
Results of inclusion vs. exclusion of quadratic terms in the Linear Mixed-Effects Model for hybridity ( $HYB > 0$ ). Model 1 refers to the model including all independent factors/variables, while Model 2 refers to the model including quadratic terms of factors elevation (a) and longitude (b), respectively.

	df	AIC	BIC	logL	deviance	$\chi^2$	$\chi^2$ df	P value
<b>(a) Inclusion (Model 2) vs. exclusion (Model 1) of the quadratic term elevation<sup>2</sup></b>								
Model 1	12	-1.9002	38.878	12.950	-25.900			
Model 2	13	-3.0686	41.107	14.534	-29.069	3.1684	1	0.07508
<b>(b) Inclusion (Model 2) vs. exclusion (Model 1) of the quadratic term longitude<sup>2</sup></b>								
Model 1	12	-1.9002	38.878	12.950	-25.900			
Model 2	13	0.0547	44.231	12.973	-25.945	0.0451	1	0.8318

factors were removed and the procedure was iterated. This process was repeated until only significant variables were left in the model. For the *lmer* procedure, the same approach was chosen. In contrast to the *glmer* procedure, where adding factor interactions would inflate the complexity of the model, the interaction term of latitude and longitude (*lat:lon*) was enclosed in the *lmer* modelling after model simplification to test for a better fit of the model.

As done with hybridisation index  $HI_{her}$ , the hybridity index  $HYB$  was subjected to a Linear Mixed-Effects Model (*lmer*). Again, only individuals with  $HYB > 0$  were included in the model fitting and the resulting model was fitted by step-wise elimination of insignificant factors. Here, also regression models with a quadratic function of some factors (*elevation*, *longitude*) were tested to improve the model and account for an expected concentration of hybrid genotypes at intermediate elevations or intermediate geographical (longitudinal) positions. The default model and quadratic model were compared with each other by the likelihood-ratio-test [command: *anova()*] in R v.2022.07.2 (R Development Core Team, 2009–2022).

**Eco-climatological niches and modelling of potential ranges.** – The actual distribution of the two species in Europe was inferred from indications of localities for purebred individuals given in Herborg (1987); Oberprieler (1994); Hodálová & Marhold (1996); Hodálová & Valachovič (1996); Hodálová (1999), and Hodálová & Kochjarová (2006), which were georeferenced using GOOGLE EARTH PRO v.7.3.4.8642. Subsequently, R v.2022.07.2 (R Development Core team, 2009–2022) and the R package ‘raster’ v.3.6 (Hijmans, 2022) was used to extract locality-specific values for 19 eco-climatic variables from WorldClim version 2.1 climate data for 1970–2000 and a spatial resolution of 30 s (<https://www.worldclim.org/data/worldclim21.html>). A principal component analysis (PCA) was performed with PAST v.4.15 (Hammer et al. (2001) to reduce complexity and illustrate eco-climatological niches of the two taxa. Potential distribution ranges for *S. hircynicus* and *S. ovatus* were inferred using MAXENT v.3.4.4 (Philips et al. 2021) with subdivision of the original dataset into six partial datasets for cross-validation and calculating the final model by averaging probabilities of occurrence across these six sub-models (Radosavljevic et al. 2014). For testing for significance of niche overlap, the R package ‘ENMTools’ (Warren et al. 2021) was used to calculate Schoener’s *D* (Schoener 1968) and Warren’s *I* (Warren et al. 2008) based on the comparison of empirical and simulated data.

### 3. Results

**Hybrid indices.** – From the 721 plants sampled, 706 hybrid indices and hybridities in 94 different plots were inferred. The remaining 15 samples were discarded because of problems during PCR reactions and/or amplicon digestion. Hybridisation index  $HI_{her}$  and hybridities  $HYB$  for all 706 individuals are given in Table S2 in the Supplementary Material; histograms of the two parameters are illustrated in Fig. 2 and box and whisker plots for each sampled population in the five regions are given in Fig. 3, where populations within regions are arranged according to their geographical longitude. In summary, there is a tendency observable for populations in the western and central part of the Bavarian Forest NP (regions 1 and 2), along with populations in regions 4 and 5, for exhibiting low  $HI_{her}$  values typical for *S. ovatus* or hybrids and backcrosses towards that species, while many populations of region 3

**Table 3**

List of the 19 Bioclim variables (WorldClim) used in the eco-climatological niche reconstructions of the present study. Mean values (and standard errors) are given for *Senecio hercynicus* and *S. ovatus* based on the analysis of 998 georeferenced herbarium specimens, together with factor loadings (PC 1 and PC 2) onto the first two principal components of a principal component analysis (PCA). Significant (t tests corrected for unequal variance in the two taxa and Bonferroni corrected for multiple testing with a significance level of  $P < 0.0026$ ) are given in bold.

		<i>S. hercynicus</i> (SE)	<i>S. ovatus</i> (SE)	PC 1	PC 2
		$n = 288$	$n = 710$		
<b>Bioclim01</b>	Annual Mean Temperature	<b>5.19 (0.13)</b>	<b>7.34 (0.06)</b>	0.27	-0.13
<b>Bioclim02</b>	Mean Diurnal Range (Mean of monthly (max temp - min temp))	<b>7.61 (0.08)</b>	<b>8.30 (0.03)</b>	0.14	0.33
<b>Bioclim03</b>	Isothermality (BIO2/BIO7) (* 100)	<b>30.8 (0.19)</b>	<b>32.1 (0.07)</b>	0.07	0.27
<b>Bioclim04</b>	Temperature Seasonality (standard deviation *100)	<b>640 (2.17)</b>	<b>665 (1.49)</b>	0.21	0.24
<b>Bioclim05</b>	Max Temperature of Warmest Month	<b>18.6 (0.15)</b>	<b>21.4 (0.08)</b>	0.30	-0.02
<b>Bioclim06</b>	Min Temperature of Coldest Month	<b>-6.03 (0.16)</b>	<b>-4.45 (0.07)</b>	0.18	-0.31
<b>Bioclim07</b>	Temperature Annual Range (BIO5-BIO6)	<b>24.6 (0.12)</b>	<b>25.8 (0.07)</b>	0.18	0.32
<b>Bioclim08</b>	Mean Temperature of Wettest Quarter	<b>7.73 (0.38)</b>	<b>12.77 (0.21)</b>	0.18	0.25
<b>Bioclim09</b>	Mean Temperature of Driest Quarter	2.24 (0.35)	2.19 (0.15)	0.01	-0.36
<b>Bioclim10</b>	Mean Temperature of Warmest Quarter	<b>13.1 (0.14)</b>	<b>15.6 (0.07)</b>	0.28	-0.08
<b>Bioclim11</b>	Mean Temperature of Coldest Quarter	<b>-2.43 (0.12)</b>	<b>-0.71 (0.06)</b>	0.21	-0.23
<b>Bioclim12</b>	Annual Precipitation	<b>1179 (19.2)</b>	<b>1045 (11.5)</b>	-0.31	0.06
<b>Bioclim13</b>	Precipitation of Wettest Month	<b>137 (2.18)</b>	<b>128 (1.54)</b>	-0.28	0.16
<b>Bioclim14</b>	Precipitation of Driest Month	<b>69.9 (1.31)</b>	<b>58.8 (0.68)</b>	-0.30	-0.08
<b>Bioclim15</b>	Precipitation Seasonality (Coefficient of Variation)	<b>22.5 (0.53)</b>	<b>25.3 (0.35)</b>	0.04	0.35
<b>Bioclim16</b>	Precipitation of Wettest Quarter	<b>383 (6.24)</b>	<b>355 (4.42)</b>	-0.27	0.17
<b>Bioclim17</b>	Precipitation of Driest Quarter	<b>231 (4.27)</b>	<b>195 (2.31)</b>	-0.30	-0.09
<b>Bioclim18</b>	Precipitation of Warmest Quarter	348 (6.07)	340 (4.51)	-0.22	-0.26
<b>Bioclim19</b>	Precipitation of Coldest Quarter	<b>273 (6.29)</b>	<b>219 (3.00)</b>	-0.28	-0.18
	<b>Variance explained</b>			<b>43.5</b>	<b>26.7</b>

(eastern part of Bavarian Forest NP and adjacent parts of Šumava NP) show clear signals of pure *S. hercynicus* or backcrosses towards that species (Fig. 3A). In terms of hybridity *HYB* (Fig. 3B), all of the populations with a hybridisation signal tend to be dominated by backcrossed individuals, while F1 hybrids (or genotypes close to a F1 constitution) are rare.

**Regression model fitting.** – The Generalized Linear Mixed-Effects Model (*glmer*) for the subsample of only purebred individuals with  $HI_{her} = 0$  (i.e., pure *S. ovatus*;  $n = 345$ ) or 1 (i.e., *S. hercynicus*;  $n = 143$ ) was run and simplified four times until it only delivered significant contributions from independent variables. Finally, only longitude and latitude were left with significant  $P$  values below 0.05 (Table 1a). The subsample of hybrid genotypes with  $0 < HI_{her} < 1$  ( $n = 218$ ), which were subjected to the Linear Mixed-Effects Model (*lmer*), showed already in the first run only longitude with a significant  $P$  value, but still latitude was taken into account for the next step because its significance was close to 0.05. However, including the interaction between longitude and latitude did not lead to a significantly better model and was dropped consequently. Finally, the simplified model with only longitude and latitude fitted the model with a significant  $P$  value (Table 1b).

The Linear Mixed-Effects Model (*lmer*) for individuals with  $HYB > 0$  showed only longitude being significant in the full model (Table 1c). The likelihood-ratio-test between the regression model with the quadratic *elevation* term and the model without the squared term showed no significant improvement by adding the quadratic term (Table 2a). Equally, the model including the squared *longitude* term did not produce a better fitted model in contrast to the model with all factors (Table 2b). This indicates that there is no hybrid zone, neither along an elevational nor along a geographical (longitudinal) gradient, that would be characterised by an overrepresentation of hybrid genotypes at intermediate elevations or longitudinal positions.

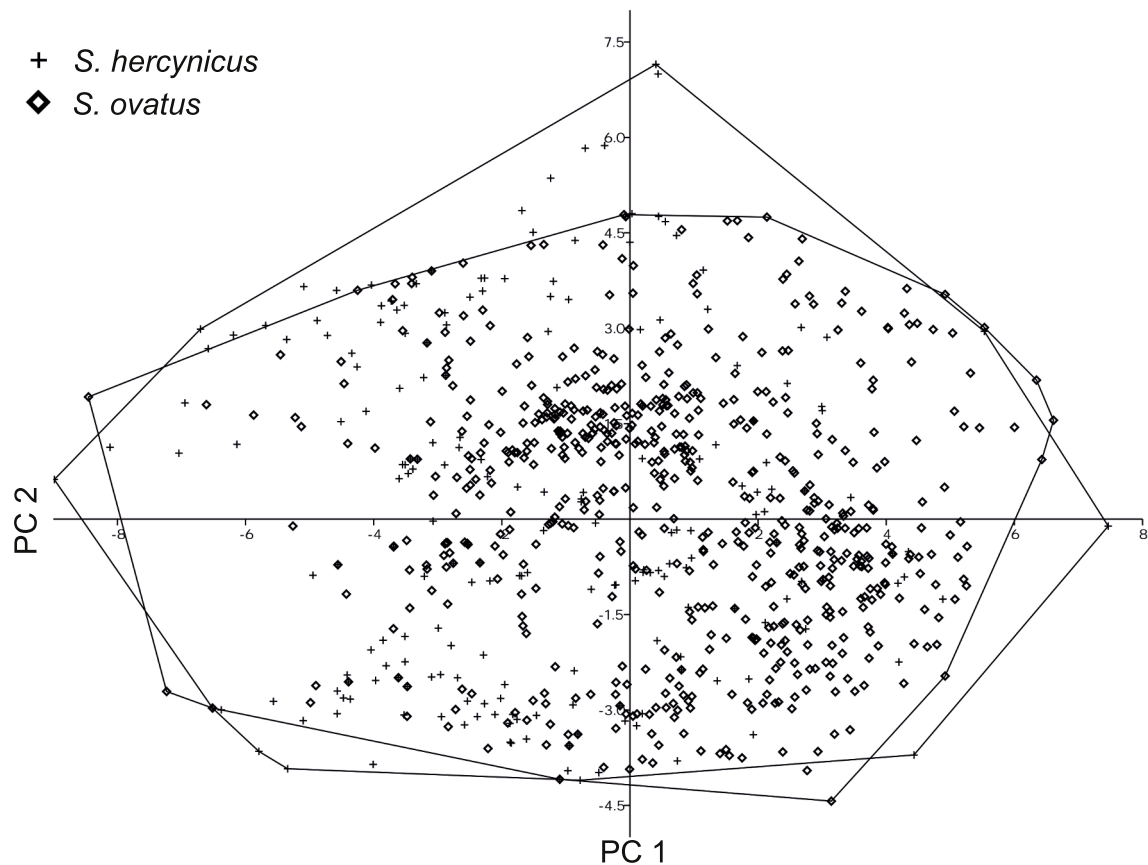
**Eco-climatological niches and modelling of potential ranges.** – We were able to gain geo-referenced presence data and values for the 19 eco-climatological variables for 998 representatives of *S. hercynicus* (288 data points) and *S. ovatus* (710 data points). While significant differences between the two species are seen in 17 out of the 19 variables (Table 3), in further analyses the eco-climatological contrast between them diminishes: Fig. 4 shows the ordination of data points based on a principal component analysis (PCA, further results summarised in

Table 3) and demonstrates the complete overlap of the multivariate habitat niches of *S. hercynicus* and *S. ovatus*. Together with maps showing the potential distribution ranges of the two taxa (Fig. 5) and the results of statistical testing for niche overlap with non-significant values both for Schoener's  $D$  and Warren's  $I$ , the lack of a significant differentiation between the two taxa in eco-climatological terms is corroborated. While *S. ovatus* exhibits a broader climatological niche compassing also low-elevation habitats, the high-elevation habitats typical for *S. hercynicus* are also indicated for the former species with high probabilities for its occurrence (Fig. 5).

#### 4. Discussion

Our present study based on the genetic characterisation of 706 plant individuals from 94 populations of *Senecio hercynicus* and *S. ovatus* and their hybrids in the Bavarian and Bohemian Forest using a PCR-RFLP approach based on a sequence difference in nrDNA ITS1 revealed a pattern of intensive introgressive hybridisation throughout the study area. Regression analyses aiming at geographical and eco-climatological explanations for distribution patterns of purebred individuals/populations ( $HI_{her} = 0/1$ ), the direction of hybridisation ( $0 < HI_{her} < 1$ ), and the hybridisation intermediarity (*HYB* index) did not support any significant determinants except the geographical position of the surveyed stands. The hybridisation and introgression gradient observed runs parallel with longitude (and latitude), with (south)western populations tending towards the *S. ovatus*, and (north)eastern ones towards the *S. hercynicus* side of a continuous spectrum, most of stands with purebred *S. hercynicus* being detected in the easternmost parts of Bavarian Forest NP and adjacent parts of Šumava NP.

With its demonstration of the intensive introgressive hybridisation between the two *Senecio* species, the present study corroborates previous genetic analyses of mixed stands of the two taxa in the Bavarian Forest region based on AFLP fingerprinting (Oberprieler et al. 2010, 2015; Bog et al. 2017a). In contrast of the mentioned studies based on multi-locus genotyping, however, the present survey relied solely on a single-nucleotide polymorphism (SNP) in the ITS1 region of the nuclear ribosomal repeat (nrDNA). On the one hand, this allowed a fast determination of the hybrid status for a large number of individuals; however, on the other hand, this approach is not capable of allowing



**Fig. 4.** Ordination of the 998 georeferenced accessions of *Senecio hercynicus* (crosses) and *S. ovatus* (diamonds) on the first two axes of a principal component analysis (PCA) based on 19 eco-climatological variables (loadings of variables are given in Table 3). While PC axis 1 accounts for 43.5 % of the total variation, PC axis 2 accounts for 26.7 %.

comprehensive statements concerning the overall constitution of the genome. Additionally, the multi-copy nature of the nrDNA tandem repeat and the observed homogenisation of this region by processes of concerted evolution (Alvarez and Wendel 2003) may be considered further disadvantages of the present study. However, the latter point (concerted evolution) may only influence and disturb analyses aiming at larger time-scales (i.e., phylogenetic inference), while the former one (multi-copy nature) may be seen as even advantageous for the aim of the present study: while an F1 hybrid may exhibit a fifty-fifty mixture of two species-specific ITS1 motives, recombination events in this region in subsequent hybrid and backcross generations will lead to the incremental genetic assimilation towards the one or the other parental genotype. As a consequence, the ratio of the two species-specific ITS motives inferred through a PCR-RFLP procedure could be viewed upon as an easily established, fast, and accurate determination of the genetic hybrid and backcross status of an individual. Previous usage of this marker system for the corroboration of the F1 hybrid status of crossing products of *S. hercynicus* and *S. ovatus* produced for transplantation and food-choice experiments (Bog et al. 2017, 2017b) supported these assumptions by showing the expected fifty-fifty mixture of the two parental ITS1 motives.

Owing to the eco-climatological preferences of the two hybridising *Senecio* species of the present study – with *S. hercynicus* limited to the montane and subalpine belts of the Central European highlands, while *S. ovatus* exhibiting a broader ecological amplitude by growing at elevations between the upland and subalpine belts – limitation of sampling populations for the present study to elevations above 900 m a.s.l. should have favoured findings of pure stands of the former species. However, over large parts of the area covered, hybridisation and even backcross

signals towards *S. ovatus* are the dominating patterns observed. This is in strong agreement with findings of previous studies by Oberprieler et al. (2015) and Bog et al. (2017a), who observed the intensive introgressive mixture of the two taxa along elevational transects between 650 m a.s.l. and 1350 m a.s.l. in the western part of the Bavarian Forest NP (regions 1 and 2 of the present study). The impossibility to find purebred *S. hercynicus* individuals in these studies, along with demonstrating that climatologically mediated, divergent selection in mixed stands does not favour reinforcement of species boundaries, therefore led to the pessimistic judgement of *S. hercynicus* being ‘lost in the hybridisation vortex’ (Bog et al. 2017a). The enlargement of the sampled areas to the complete Bavarian Forest NP and adjacent regions of Šumava NP in the present study, however, has revealed that *S. hercynicus* still exists in purebred stands in the Bavarian and Bohemian Forest region.

In contrast to a study of the *S. hercynicus*–*S. ovatus* hybrid system in the central German Harz Mountains, where an elevational gradient with purebred *S. hercynicus* on the summit of Mt Brocken and *S. ovatus* at low elevations with hybrid swarms in between was observed (Raudnitschka et al. 2007), our present study shows that neither an elevational nor any habitat or eco-climatological factor allows prediction of the occurrence of purebred *S. hercynicus* populations. Only geographical position – and here longitude to a larger extent than latitude – was found being positively correlated with the chance of finding pure *S. hercynicus* stands (Table 1a) or hybrid swarms tending to be on the *S. hercynicus* side in genetic respects (Table 1b). This geographical pattern may be best explained by the northwest-southeast orientation of the Bavarian and Bohemian Mountain ranges, with the largest parts of the Bavarian Forest NP (especially regions 1 and 2 of the present study) comprising the south-western oriented declivities of this cordillera, while the Šumava

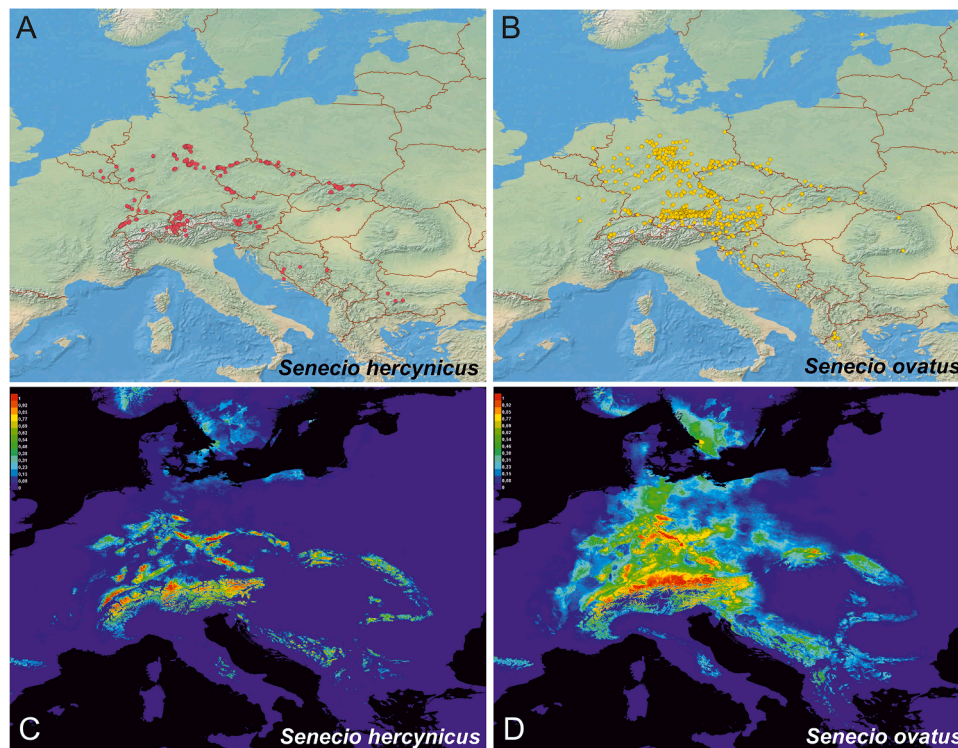


Fig. 5. Actual distribution ranges of *Senecio hercynicus* (A) and *S. ovatus* (B) based on 288 and 710 georeferenced herbarium specimens, respectively. (C, D) Potential distribution ranges of the two taxa based on eco-climatological niche-modelling.

NP (with some populations of region 3 of the present study) lies on its north-eastern slopes. While it seems that fine-grained eco-climatological differences coming with this geographical pattern do not have any significant influence on the presence of purebred *S. hercynicus* or the direction of introgressive hybridisation, coarse-gained biogeographical patterns and invasion-dynamic processes could be the clue for these results. Range shifts caused by climate change and the connected upwards shifts of species distributions (Thuiller 2007) surely favours the thermophilic *S. ovatus* over *S. hercynicus*, leading to invasions of former *S. hercynicus* habitats and (due to a shift in the phenological behaviour of *S. ovatus* towards earlier flowering times overlapping with early-flowering *S. hercynicus*) to more intense hybridisation. These processes could be expected being more intense on the south-western slopes of a mountain range than on the north-eastern declivities.

The hypothesis of a temporal contribution to the hybridisation dynamics in the *S. hercynicus*–*S. ovatus* system gains support from the regression analysis of the present study aiming at the explanation of hybridisation intermediarity as measured by our *HYB* index: showing a significantly positive influence of geographical longitude on this index (Table 1c) indicates that in the eastern part of the surveyed region, hybrid plants tend to be closer to a fifty-fifty ITS1 (F1) genotype (*HYB* → 1.0) than in the western part. This may indicate that hybridisation between the two species followed by intensive backcrossing has lasted longer in the (south)western than in the (north)eastern parts, where genetic swamping has started later due to a later invasion of *S. ovatus* into *S. hercynicus* habitats and/or due to the more pronounced phenological differences between the two species in these climatically less favoured habitats. However, the observed overlap of and non-significant difference between the eco-climatological niches of the two species (Figs. 4, 5, and Table 3) argue for a reduced importance of extrinsic, environment- or geography-mediated reproductive isolation mechanisms and shift attention towards an intrinsic one: flowering time.

It is known from both observations of mixed stands of the two species in nature (Oberprieler et al. 2010, 2016) and from common-garden experiments (Herborg 1987) that *S. hercynicus* is capable to

vegetatively develop faster than *S. ovatus* and to start flowering around 4–5 weeks earlier than its congener, allowing co-existence of the two entities in the same habitats. Only after formation of F1 hybrids caused by delayed flowering of *S. hercynicus* due to browsing or earlier flowering of *S. ovatus* caused by accelerated vegetative development motivated through habitats becoming snow-free earlier in the year, these plants with an intermediate flowering time and an unreduced fertility will form a turntable for gene flow between the two species and will lead to the formation of hybrid swarms with their complete blurring of taxonomic boundaries. The longer and more intense forestry operation history on the south-western, climatically favoured slopes of the Bohemian Mountain range (Bavarian Forest NP) as compared to the north-eastern declivities with their harsher conditions and their long-lasting isolation and reduced intensity of forestry operations caused by the ‘iron curtain’ (Šumava NP) may, therefore, be an additional explanation for the different hybridisation intensities observed in the regions of the present study.

As a consequence, owing to the lack of significant habitat differences between *S. hercynicus* and *S. ovatus*, the observed lack of selection regimes towards purebred genotypes in mixed stands and hybrid swarms along an elevational gradient (Oberprieler et al. 2015; Bog et al. 2017a), the expected shift of climate conditions towards the ‘*S. ovatus* end’, and the irreversibility of hybridisation and backcrossing once intermediate genotypes are formed, *S. hercynicus* has to be still considered being threatened by extinction through genetic swamping. It seems that we have documented here the last remnant stands of this species in the Bavarian-Bohemian region before sinking into oblivion. Measures of *in-situ* species conservation may comprise reduction of human impact leading to disturbances promoting earlier flowering of *S. ovatus* (realised on the Bavarian Forest NP side due to its status as a national park, but not on the Šumava NP side due to heavy treatments of stands of Norway spruce (*Picea abies*) against bark-beetle pests) or the removing of F1 hybrids soon after their formation; the latter being against a non-invasive strategy obligate for national parks in their original ambition and a work program of Sisyphean dimensions. Therefore,



following Kramer & Havens (2009) and Oberprieler et al. (2015) in their evolutionary argumentation, we should accept in the case of *S. hercynicus* a perspective of ‘adaptive introgression’ (Taylor and Larson 2019) leading to a new, chimeric taxon that will remain available as member of plant communities and player in complex organismal networks.

### CRedit authorship contribution statement

**Christoph Oberprieler:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Daniel Lenz:** Writing – original draft, Formal analysis, Data curation. **Pia Donhauser:** Writing – original draft, Formal analysis, Data curation. **Claus Bässler:** Writing – review & editing, Conceptualization.

### Declaration of competing interest

The authors declare no conflict of interests.

### Data availability

Data will be made available on request.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2024.152602.

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