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Investigating introgression between the novel rubber crop, *Taraxacum koksaghyz*, and its congeners across native and introduced ranges

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Abstract

Experimental crosses between the rubber dandelion, *Taraxacum koksaghyz*, and related dandelion species show that interspecific hybridization is possible. Therefore, in regions where *T. koksaghyz* is grown for rubber production and in its native habitat in southeastern Kazakhstan, introgression may occur. Introgression in both situations is investigated here. A large-scale population genetic study was conducted to determine whether *T. koksaghyz* genes are still present in the environment 66 years after its seed production in southern Sweden was terminated. Twenty-one populations of wild dandelions at distances ranging from 0.1 to 25 km around the original seed nurseries were sampled. Using ten newly developed *T. koksaghyz* specific codominant DNA markers, in total, more than 14,500 alleles were typed. Not a single *T. koksaghyz*-specific allele was found, suggesting that introgression in South Sweden is absent or extremely low. Within its native range in southeastern Kazakhstan, *T. koksaghyz* is mostly spatially isolated from related dandelion species by habitat differentiation. However, it may grow intermingled with other dandelion species at disturbed sites. Introgression at two of such disturbed locations was also investigated. From the combined analysis of ploidy level, reproduction mode, and multilocus genotypes, we infer that there is no indication of gene flow between *T. koksaghyz* and sympatric *Taraxacum* species in natural populations in Kazakhstan. Therefore, *T. koksaghyz* populations in Kazakhstan are not likely to be threatened by gene swamping caused by other dandelion species.

Introduction

More than 40% of all rubber used worldwide is natural rubber. Due to specific properties, natural rubber cannot be replaced by synthetic rubber in most of its important applications, and its global production and consumption steadily increase to exceed 13 MMT annually (2017–2019). There is a considerable interest in developing alternative rubber crops for the temperate regions (Cornish 2017) due to the vulnerability of plantations of the rubber tree, *Hevea brasiliensis* (Willd. ex A. Juss.) Muell. Arg., the only current commercial source of natural rubber (Mooibroek & Cornish 2000, Kirschner et al. 2013). Comprehensive surveys identified only a few suitable species. Guayule (*Parthenium argentatum* A. Gray) and the rubber dandelion, also known as Kazakh (or Russian) dandelion, *Taraxacum koksaghyz* Rodin (hereafter TKS), are the most important ones. TKS development programs are implemented on national (USA, China, Germany), and international (EU) scales (Stolze et al. 2017, Benninghaus et al. 2020, Ramirez-Cadavid et al. 2017, 2018, McAssey et al. 2016, Kreuzberger et al. 2016, Lin 2009, Lin et al. 2017, Luo et al. 2018), and the natural rubber research was resumed in Russia (Kuluev et al., 2019). In the present article, introgression between TKS and congeners is investigated using codominant nuclear DNA markers. Information about introgression is relevant for the conservation of TKS in its natural habitat and the possible effects that large-scale TKS cultivation in other regions can have on the native floras.

As regards the evolutionary relationships of TKS, it belongs to *Taraxacum* sect. *Ceratoidea* Kirschner & Štěpánek (2008), with two closely related sexual diploid species (*T. bicorne* Dahlst. and *T. neolobulatum*

van Soest ex Schischkin, see also Zeisek et al. 2019). There are a few agamospermous polyploid members of this section allied to the core sexuals.

TKS was discovered in 1931 in southeastern Kazakhstan, confined to a relatively small region near the border with China (including a very limited border area in Xinjiang). It is a sexual self-incompatible diploid (2n = 2x = 16), which outcrosses by insect-mediated pollen flow (Poddubnaya-Arnoldi & Dianova 1934, Kirschner et al. 2013, Ge et al. 2011). It was immediately recognized as a potential rubber crop, described as a new species, cultivated, studied, and systematic breeding started (Rodin 1933, Lipschitz 1934). In the 1930s, the domestication and agronomy of TKS were first developed exclusively in the USSR. Before World War II, achenes (one-seeded fruits) of TKS were not provided to other countries. However, in the 1938–1940 period, A. Hitler was recognized as a politically acceptable partner for sharing this strategic resource. After the Nazi invasion of the Soviet Union, other plant materials, equipment, and knowledge were stolen and used for breeding programs in Germany, Austria, and other allied countries. At that time, there was a general rubber shortage, and emergency rubber programs using TKS were set up in many countries (e.g., USA, Germany, England, Finland, Sweden), with TKS material obtained from the USSR (for details of the German program, see Heim 2008, for additional information, see Kirschner et al. 2013).

Most of the emergency rubber programs were terminated immediately after WW II before they could yield exploitable results. In Sweden, however, it continued till 1952 (Josefsson 1953). Although a few TKS samples survived in the germplasm banks (e.g., VIR in St. Petersburg), virtually no material remained from the 1930–1950 breeding programs. There was an obvious need for new plant material. The attempts by Volis et al. (2009) were unsuccessful, and it was suggested that TKS had become a rare species in its natural range due to overcollecting during Soviet times. However, this was challenged by Van Dijk et al. (2010), who stressed the importance of habitat and flowering time for sampling. A new, more representative material was collected in Kazakhstan after 2000, and it is now preserved in several seed banks of international importance.

Ecological genetic interactions between TKS and congeners

Before introducing a new agricultural crop, it is important to study potential environmental side effects, including ecological genetic interactions with local related species. Little attention has been paid to this in the TKS research. As a result of introgression, a minority species can be assimilated into the gene pool of the majority species (genetic assimilation or genetic swamping; Ward 2008; Schierenbeck 2011). There is a unilateral hybridization direction in *Taraxacum* between sexuals and apomicts: pollen-producing apomicts can fertilize sexual plants, but not *vice versa*. Introductions of *Taraxacum officinale* Wigg. have generated interspecific apomictic hybrid clones with native diploid sexuals in the USA (with a taxon referred to as *T. ceratophorum* (Ledeb.) DC.; Brock 2004) and Japan (with *T. japonicum* Koidz.; Matsuyama et al. 2018, see also Kandori et al. 2009). Another significant reproductive interaction is the pollen donor effect in which pollen from triploid *Taraxacum* can lead to the breakdown of the self-incompatibility system of sexual dandelions, as demonstrated in *T. officinale* (Morita et al. 1990; Tas &

van Dijk 1999). Brock (2004) has shown that this also occurs in interspecific pollination: pollen from apomictic 3x *T. officinale* induced selfing on a self-incompatible sexual species of *T.* sect. *Borealia* Hand.-Mazz. As a result, self-incompatible sexual species will be less prone to introgression and persist longer.

For the sake of completeness, we have to mention possible interactions between TKS and another sexual species, *T. bicorne*. Experimental crosses (in both directions) were highly successful (Zeisek et al. 2019), and natural hybridization therefore should be taken into consideration. In southeastern Kazakhstan, these two species are spatially and ecologically separated (with the minimum distance between populations of *T. bicorne* and TKS being only ca. 50 km but about 800 m as regards the altitude). The climatic optimum of *T. bicorne* is 13.7°C mean annual temperature and 322 mm annual rainfall (near Samarqand, Uzbekistan), while that of TKS is 1.9°C and 485 mm, respectively (Kirschner et al. 2018).

The crossability and sympatry determine, to a large extent, the potential for introgression between related taxa. In this paper, we investigated two cases of sympatry between TKS and other *Taraxacum* species: 1, in its native range in southeastern Kazakhstan, and 2, in its introduced range, in Sweden, where TKS was grown as a new rubber crop between 1943 and 1952. First, we discuss the potential for introgression based on what is known from experimental crosses between TKS and congeners in these regions. After that, we discuss the sympatry/parapatry, the habitats in which TKS occurs, and the introduction history in Sweden.

Experimental TKS interspecific crosses

An early study (Koroleva 1939) outlined the potential of experimental crosses between TKS and other species as a method to increase rubber yield. Within the framework of the European projects EU-PEARLS and DRIVE4EU, a broad program of extensive crossing experiments with TKS and a wide range of diploid sexual and polyploid agamospermous dandelions was performed (Kirschner, unpublished).

Crosses of TKS with triploid agamospermous *T. brevicorniculatum* (see below) initially yielded less than five achenes per capitulum. About 60% of the achenes were pure TKS through forced selfing caused by a mentor pollen effect (Tas & van Dijk 1999, Nishida et al. 2014). In contrast, crosses with an undescribed tetraploid apomict, yielded about ten achenes per capitulum, all hybridogenous. In the BC2 generation, the number of achenes per capitulum increased to 10–12 achenes in the former case and ca. 25 in the latter. Therefore, the hybridization program proved the penetrability of the ploidy and reproduction system barriers. Zeisek et al. (2019) described the results of reciprocal crosses between TKS and sexual diploid *T. bicorne* Dahlst. TKS can also be artificially crossed with more distantly-related dandelion species originating from outside its native geographic range, such as the common dandelion *T. officinale* (hereafter TO), both at the diploid and the polyploid level (Iaffaldano et al. 2018, P. J. van Dijk, unpubl., J. Kirschner, unpubl.).

Potential for introgression in the natural habitat of TKS

There are indications that natural hybridization between TKS and apomictic relatives has resulted in the formation of new hybridogenous apomictic lineages in the past. During the post-2000 field studies, several agamospermous species were found in the region of TKS occurrence, showing a certain morphological resemblance to TKS. This was primarily *Taraxacum brevicorniculatum* V. Korol., an agamospermous triploid (2n = 3x = 24) that probably originated through alloploidy with TKS involved (P. J. van Dijk, unpubl., Kirschner et al. 2013, and Zhang et al. 2017). In fact, Kirschner et al. (2013) showed that TKS seeds that were registered pre-2000 in worldwide seed banks belonged to this apomictic species. In *Taraxacum*, a geographical parthenogenesis model (Hörandl 2006) is encountered quite frequently, and many sexual diploids gave rise to a series of closely related agamospermous taxa through alloploidy (Kirschner & Štěpánek 1996, Kirschner et al. 2020). There is another agamospermous species occurring in, or near, the distribution range of TKS and exhibiting a notable rubber content. It is an undescribed tetraploid (2n = 4x = 32), now under examination, with a high probability of the alloploid origin involving TKS (J. Kirschner, unpubl.).

Within its native geographic range in Kazakhstan, TKS is usually spatially isolated from other related dandelions by habitat differentiation (van Dijk et al. 2010; Kirschner et al. 2013, Kirschner et al. 2018). However, at some disturbed sites in the Saryzhaz Basin, TKS grows together with related dandelion species, and interspecific gene flow could occur. Breakdown of habitat isolation followed by gene flow between TKS and other dandelion species may have negative consequences for the conservation of TKS (genetic swamping effect, cf. Schierenbeck 2011). In the vicinity of Sarydzhas, two of such disturbed locations were studied. Here introgression would be detectable if TKS populations were not fixed for TKS-specific DNA markers.

Potential for introgression after the introduction of TKS as a new rubber crop outside its native range

The Swedish cultivation of TKS is one of the longest and geographically best documented cultivations in and after WWII and therefore offers one of the best opportunities to investigate the occurrence of longer-term introgression. The history of TKS cultivation in Sweden is summarized in Joseffson (1953). Additional information was obtained from Suomela (1950). Sweden obtained a small amount of TKS seed in 1942 and 1943 from the USSR. These batches were immediately tested in Ultuna by the School of Agricultural Sciences (near Uppsala, 59.82 N, 17.66 E), on experimental farms and experimental stations, as well as on individual farms in various parts of Sweden. This mainly concerned cultivation-technical research. The journal *Nature* (Anonymous 1943), citing the Anglo-Swedish Review, reported that approximately 300 kilograms of TKS seed were expected to be harvested by the end of 1944. Moreover, 230 hectares of land would have been sown with TKS. Given that later in the more rational program, 150 kg of seed was collected over four years in several seed nurseries, these projections are unrealistic, and the surface area of TKS cultivation in those years must have been much smaller. Indeed, in 1945, only 10 hectares of TKS were grown throughout Sweden, at 150 localities, ranging from Skåne in the South to Northern Norrland most north (Suomela 1950). The average plot size was thus only 0.07 hectares.

Since it was clear that significant improvements in TKS cultivation and plant material were needed to increase the rubber yield from 1947 onwards, research in Sweden was streamlined. Agronomic research was conducted at Flahult experimental farm (near Taberg, 57.69 N; 14.13 E). The agricultural Engineering Institute performed TKS cultivation experiments at Ultuna in connection with the construction of machinery for the mechanization of cultivation.

The Swedish Seed Association (SSA) was responsible for TKS selection for higher rubber yields and the production of seeds. At the famous Plant Breeding Institute at Svalöf (55.93, 13.10) TKS was bred for increased rubber content of the roots. The seed production took place mainly at the agricultural school at Tomelilla (55.32 N; 13.56 E) and on the Olsgård farm near Hammenhog (55.29 N; 14.07 E). Other seed nurseries were on Oland (56.67 N; 16.62 E) and at Singeshult near Lidhult (56.74 N; 13.36 E). On the Olsgård farm, TKS was also grown for pre-selection of plant material for further rubber and root size selections at Svalöf (in some years, 20,000 plants) and for evaluation of materials that had been selected at Svalöf.

The promising results (rubber content tripled to 15% in six years, Josefsson 1953) convinced the Swedish government to continue the program if the rubber industry was also willing to invest. The rubber industry was not prepared to do this, probably due to the changed circumstances, with *Hevea* rubber again widely available and the promise of synthetic rubber. Therefore the SSA was forced to terminate the TKS rubber program.

Investigating the presence of TKS genes in current wild dandelion populations can provide insight into the possible long-term population genetic effects of the future introduction of TKS as a new rubber crop. In southern Sweden, the native dandelions belong mainly to *Taraxacum* section *Taraxacum* (*T. officinale* s. lat.; TO), and less commonly to *T*. section *Hamata* H. Øllgaard, *T*. section *Erythrosperma* (Lindb. fil.) Dahlst. and *T*. section *Palustria* (Lindb. fil.) Dahlst. These taxa are obligate apomicts and almost exclusively triploid, seldom tetraploid. There is evidence that TKS can hybridize with *T. officinale* in the field. Małecka (1971) found natural hybrids between 2x TKS and 3x apomictic TO growing between their parental species in the botanic garden at Jena, Germany (although there might be certain doubts about the identity of the plants). Hybridization experiments were carried out by Koroleva (1940), and Iaffaldano et al. (2018) found rare hybrids between TKS in plantations and wild agamospermous dandelions in Ohio. Figure 1 summarizes the possible reproductive interactions, based on artificial crosses and natural introgression studies.

Based on the results from experimental crosses described above, it seemed possible that introgression could have taken place, mainly by producing first-generation and BC apomictic hybrids. We were interested in finding out whether, after 66 years, introgression was detectable. We looked for TKS morphology and TKS-specific DNA markers. Dandelion seeds are particularly well adapted to wind dispersal, making long-distance dispersal possible (Darwin 1859, Cummins, 2018). Spreading pure or hybridogenous TKS seeds can become a secondary source for introgression after establishment. Tackenberg et al. (2003) showed in computer simulations that 99.5% of all the dandelion seeds landed

within 10 meters from the source, 0.05% were dispersed more than 100 meters, and 0.014% of the seeds could disperse over distances of more than 1 kilometer. In the county of Skåne, the Olsgård farm and the agricultural school at Tomelilla were the centers of TKS cultivation, and here, the chances of finding traces of TKS introgression are maximum. Given the good dispersal capacity of dandelion seeds, we sampled at a wide scale, at different distances from these historical seed nurseries.

Materials and Methods

Population sampling

A. TKS population sampling in southeastern Kazakhstan. At two sites in Kazakhstan, with sympatric populations of TKS and other dandelions, where hybridization and introgression were potentially possible, dandelions were sampled as roots in June 2014 by J. Kirschner and J. Štěpánek. These sites were located in the Almaty Region, Saryzhaz Basin, between the Terskei Alatau (Gory Basulytau) and the Ketmentau, Saryzhaz (Sarydzhaz), see also Fig. 2.

Location A: Saryzhaz, south of the bridge across the Kegen River (42°56.050 N, 79°37.034 E). Dry to mesophilous subsaline steppes along the road between Saryzhaz and Komirshi, c. 4 km N of Saryzhaz. The altitude of this site is 1883 m. The following plants were included in this study: Nine probably triploid apomictic plants (#57); two triploid apomicts (#58) and two diploid TKS plants (#59). All belong to the same local mixed population.

Location B: Saryzhaz, towards Komirshi, north of the bridge across the Kegen River (42°59.416 N, 79°35.919 E). Dry to mesophilous subsaline steppes along the road between Saryzhaz and Komirshi, about 100–200 m north of the bridge across the Kegen River. The altitude at this site is 1861 m. The following plants were included in this study: Four TKS plants (#61), two *T. brevicorniculatum* plants and similar polyploid plants (#62); six *T. brevicorniculatum*-like plants (#63); and one putative hybrid, seemingly an intermediate between TKS and *T. brevicorniculatum*, but closer to TKS (ploidy unknown; #64). All belong to the same local mixed population. Near the latter locality, there are permanent plots with TKS (the method of TKS documentation is shown on Fig. 3).

B. Taraxacum sp. population sampling in southern Sweden.

In total, 21 populations were sampled in May 2016 at different distances from the former seed production sites (see Figs. 4, 5 and 6). Table 2 lists the names of sampling sites and Fig. 5 shows the distances to the nearest former seed production field (for GPS coordinates and site characteristics, see Table 2). At Hammenhög, the Olsgården farm still exists. The land surrounding this farm consists mainly of arable land, and populations of dandelions are relatively sparse. Three populations were sampled within a 2 km distance to the Olsgården farm and three within a 3 km distance. At Tomelilla, the Agricultural School had been converted into a Folk High School, and the cultivation fields no longer exist as agricultural fields. Nevertheless, dandelions could be sampled in the neighborhood of the school. Five populations were sampled within a 2 km distance from the former Agricultural School. Altogether, eleven

sites were located within 2.5 kilometers of the previous TKS seed nurseries; ten were outside the 2.5-kilometer distance. The two most distant locations were A (Ystad) and U (West of Skällbäck) at 12 and 25 km from the former seed production sites. These two populations can be considered as references for pure TO populations; no TKS introgression is expected at these sites. To detect low levels of introgression, the highest sampling density was close to the nurseries. At three locations nearest to the former seed nurseries, 48 plants were sampled; at the other 18 sites, 24 plants were sampled. Five to six 5 mm diameter leaf disks (Fig. 4D) were taken in the field and kept at ambient temperature in microtubes containing silica gel. DNA of 576 sampled plants was extracted at the KeyGene laboratory in the Netherlands, according to the protocol of Doyle and Doyle (1990).

Table 1
TKS seed harvests (kg/hectare) at the Hammenhög and Tomelilla seed nurseries between 1947 and 1950 (Josefsson 1953). The size of the Hammenhög and Tomelilla plots was 2000 m^2; therefore actual seed harvests were one fifth of these numbers.

	Hammenhög		**Tomelilla**	
	1st year plants	***2nd year plants***	***1st year plants***	***2nd year plants***
1947	43.4			
1948	14.4	16.6		
1949	5.1	18.3	21.3	
1950		22.5		9.8
Total	120.3		31.1	

Development and application of DNA markers

Single Nucleotide Polymorphisms (SNPs) between TO and TKS were mined in genome sequences (see below). To screen a large number of plants effectively, KASP™ (Kompetitive Allele Specific PCR) assays were developed for SNP detection. Based on 50 bp sequences immediately upstream and downstream of the SNP, KASP™ assays were designed by LGC, Biosearch Technologies (https://www.lgcgroup.com). KASP™ markers are codominant and can be used to genotype different types of heterozygotes in polyploid plants, which are common in dandelions. KASP™ assays were run in a Roche LightCycler® 480 at KeyGene. Allele-specific fluorescence signals were processed and plotted in bi-plot graphs, using an Excel macro that was developed in-house.

Two marker sets were developed. The first consisted of genes that were involved in the rubber biosynthesis pathway. Based on the sequences of the Bacterial Artificial Chromosome libraries of TO (developed in the EU-project APOTOOL; P. J. van Dijk, unpublished) and of TKS (developed in EU-PEARLS, P. J. van Dijk, unpublished), SNPs between TO and TKS were identified. Sequencing of the cis-prenyl transferase BACs of TO and TKS indicated that TO possessed one CPT gene (CPT1) and TKS two CPT

genes (CPT2 and CPT3). These genes are nearly identical in DNA sequence, making the development of gene- and species-specific KASP™ markers difficult. A CPT1 KASP™ assay could be developed that distinguishes CPT1 of TO from CPT2 of TKS. The development of KASP™ markers for other rubber biosynthesis genes was less complicated. Rubber biosynthesis gene sequences published in Schmidt et al. (2010) and Epping et al. (2015) were used. In Table 3 the genes of KASP™ marker set I are listed. To test for species specificity, the KASP™ assays were run on a panel of ten diploid sexual TKS and ten diploid sexual TO plants originating from pure populations in Kazakhstan and Switzerland, respectively. All KASP™ markers showed a species-specific polymorphism in this panel.

The second set of species-specific markers was developed from the TKS and TO Expressed Sequence Tag (EST) libraries at The Compositae Genome Project website (http://compgenomics.ucdavis.edu/). Genes were identified that had SNPs between the TKS and the TO EST sequences. To avoid sequencing errors, only highly expressed genes with many transcripts were selected. In contrast to marker set I, these gene products are not involved in the rubber biosynthesis pathway. KASP™ assays were developed for 13 genes and validated for species specificity on the TKS-TO test panel (see above). Six markers were species-specific in the panel and were chosen for introgression studies (see Table 3). The flanking sequences used for the KASP™ assay design are listed in Table 3.

Results

The southeastern Kazakhstan study

Table 6 shows the KASP™ genotyping results at the two locations where TKS grows sympatrically with congeners. Most markers could be scored unambiguously. For eight of the nine markers, the six TKS plants are homozygous for a different SNP than the related polyploid apomicts. This is the case for both disturbed sites. Only the REF marker is heterozygous in most TKS plants, but in a previous screening of a wider TKS germplasm, it was already noticed that this marker is not entirely TKS-specific (P. J. van Dijk, unpublished). However, the overall conclusion is that there are no signs of introgression of alien genes into TKS at these sites. The two marker sets both divide the apomicts into three different clonal (apomictic) genotypes. The morphologically suspected putative TKS hybrid (#68) did not contain TKS-specific DNA-markers.

Table 3
Gene and EST sequences that were used for the development of species-specific markers. The *Arabidopsis* homologs of the Taraxacum ESTs were identified by protein BLAST against The Arabidopsis Information Resource TAIR10 genome release.

Set	Gene / EST	Protein function	TAIR10 hit	Significance
I	CTP1	Cisprenyltranferase 1		
I	RTA	Rubber transferase activator		
I	SRPP5	Small rubber particle protein 5		
I	REF	Rubber elongation factor		
II	CTO_M1_Contig179	Protein of unknown function, DUF538	AT2G04520	4.8e-057
II	CTO_M1_Contig2608	Nucleic acid-binding, OB-fold-like protein	AT5G19860	5.6e-046
II	CTO_M1_Contig3593	Vacuolar H+-ATPase subunit E isoform 3	AT1G64200	6.5e-062
II	CTO_M1_Contig5704	Unknown protein	AT1G79660	2.8e-014
II	CTO_M1_Contig6303	Plasma membrane intrinsic protein 1C	AT1G01620	4.9e-133
II	CTO_M1_Contig6322	no At hit	-	-

The southern Sweden study

At none of the 21 locations that were surveyed, dandelion plants were observed with a pure TKS morphology or with obvious TKS morphological traits (horned involucral phyllaries, the leaves' thickness, bluish color of the leaves, type of serration of the leaf, etc. Kirschner et al., 2013). Observations were made by one of us (J.K.), who is an experienced *Taraxacum* taxonomist. There is no morphological indication that TKS established permanent populations outside the cultivation fields, nor introgression based on morphological traits.

Figure 7 shows a KASP™ bi-plot, typical for nine of the ten assays. The TKS allele's fluorescent signal is plotted on the Y-axis, the fluorescent signal of the TO allele is plotted on the X-axis. The ratio of the sampled plants' fluorescent signals was in nearly all cases close to the TO control on the X-axis. Figure 10B shows a similar bi-plot but with one putative hybrid plant. Figure 10C shows the very different bi-plot for assay CTO_M1_Contig2608-372. All samples of this assay seem heterozygous for the TKS and TO alleles.

Table 5 shows the overall number of genotypes of the nine KASP™ assays, including the dropouts. For all KASP™ assays almost all sampled plants were homozygous for the TO-specific allele. Only seven putative heterozygous (hybrid) genotypes were scored. Six of these seven showed poor signals and were likely to be unreliable. One CPT1 heterozygote, however, gave a clear signal. This plant was sampled

immediately adjacent to the Olsgård TKS seed production farm and, therefore, very interesting. However, in a repeated assay, the hybrid CPT1 genotype could not be confirmed; the specific plant was homozygous for the TO allele. Probably this assay was contaminated in the laboratory. The other six heterozygous samples with weak signals also could not be confirmed in a repeated assay. By dilution of template DNA concentrations for most dropout samples, several KASP™ markers could be scored. These, however, were always TO alleles, and there is no indication that there was a species-specific bias in the dropouts (results not shown).

A total of 4884 KASP™ assays have been successfully run (see Table 6), each genotyping a single locus. Assuming that the native dandelions are triploid, this is equivalent to 14,652 alleles. Among this number, not a single TKS-specific allele could be confirmed. Restricting this analysis to the eleven populations within 2.5 km distance from the historical seed nurseries, it can be concluded that among 336 plants, or 9072 genes analyzed, not a single TKS-specific gene variant was found.

Table 6
Genotype numbers of the Skåne samples. Heterozygotes marked with an asterisk could not be reproduced.

KASP marker	TKS homozygote	Putative Heterozygote	TO homozygote	Drop outs
CPT1	0	1*	545	31
RTA	0	1*	512	64
REF	0	5*	496	80
SRPP	0	0	505	72
CTO_M1_Contig179-397	0	0	566	10
CTO_M1_Contig2608-372	0	563	2	11
CTO_M1_Contig3593-566	0	0	564	12
CTO_M1_Contig5704-271	0	0	570	6
CTO_M1_Contig6303-344	0	0	568	8
CTO_M1_Contig6322-430	0	0	558	18

Discussion

Hybridization potential of TKS from a microevolutionary perspective

As mentioned in the introduction, an extensive scheme of interspecific experimental hybridization with TKS as a mother plant was developed in different research programmes. It turns out that there is a very strong reproductive barrier between TKS and apomictic dandelions. However, there are at least two polyploid apomicts where the triploid and tetraploid genomes contain one or several chromosome sets of TKS. This is proof of the fact that occasionally such hybrids may occur, and, in a very low number of cases, the occasional TKS hybrids may give rise to stabilized apomictic polyploid species (Kirschner et al. 2013).

In its native geographic range, usually TKS is usually restricted to particular habitats, and this ecological isolation will also result in spatial isolation. In such stands, TKS is the only dandelion species. For example, a situation found in the region of the Tuzkol salt lake (Fig. 2). Although other dandelions occur in the TKS vicinity stands [(for instance, a sexual diploid, but late flowering *T. besarabicum* (Hornem.) Hand.-Mazz.]), there is a phenology barrier, and they are probably outside the effective pollination distance. At semi-natural pastures with a moderate disturbance, TKS may be dominating but coexisting with other dandelions, mostly *T. brevicorniculatum*, interspersed. Here interspecific gene flow seems possible.

However, interspecific gene flow is most likely to occur at semi-ruderal sites (along roads, tracks, etc.), usually in the area of a common occurrence of TKS, and with other dandelions at least equally common as TKS and growing mutually intermixed. However, at disturbed sites, this ecological isolation may break down, in the worst case resulting in genetic swamping by hybridization and the disappearance of TKS. To obtain a better understanding of the conservation status of TKS in its native range, dandelions at two locations were studied. At two such uncommon sites, we investigated if TKS plants were fixed for TKS-specific alleles or whether they contained alien alleles. This turned out not to be the case; even the putative morphological hybrid did not show signs of introgression. It can be concluded that in addition to ecological differentiation, other gene flow barriers restrict the introgression of non-TKS genetic material.

Lasting introgression effects from the past?

The seed cultivation of TKS in Skåne at the end of the 1940s and early 1950s offers a unique opportunity to investigate the historical invasiveness and the historical introgression of TKS genes into wild TO dandelions and to make predictions about the potential long-term effects of future TKS cultivation in areas where only apomictic triploids occur.

The seed harvest of TKS between 1947 and 1950 has been documented by Josefsson (1953, Table 1). Seeds were produced in the nurseries of Olsgården between 1947 and 1950 (4 years) (Fig. 4A) and in Tomelilla in 1949 and 1950. According to Josefsson, most seeds were picked by hand, and in dry, sunny weather, a manual harvesting machine was also used (Fig. 4B). The seed yields are in Table 1. Converted to seed numbers (assuming an average seed weight of 0.40 mg), more than 60 million TKS seeds were harvested in Olsgården and more than 15 million seeds in Tomelilla in the period from 1947 to 1951. However, many more seeds will have been produced but not harvested. Uneven ripening of achenes

made harvesting difficult, and "loads of seeds were lost" because the seeds detach easily and are carried away by the wind (Josefsson 1953: 350).

To get an idea of the amount of seeds dispersed into the environment, we can make a rough conservative estimate of the number of seeds that were produced in Olsgården and compare this to the number of harvested seeds. The nursery had an area of approximately 2000 m2, and assuming a density of 20 plants per m2, this corresponds to 40,000 plants. The plants in the photos in Josefsson are large and flower abundantly. An average of 15 inflorescences per plant and 80 florets per inflorescence is therefore a reasonable assumption, giving 125 million florets (each with a single ovule) per year. Assuming that 75% are pollinated and develop into seeds, this corresponds to 93,750,000 seeds per year. Olsgården was in operation for four years, corresponding to 375,000,000 seeds produced.

At Olsgården, a total of 60,000,000 seeds were harvested, i.e. 18% of the estimated number of seeds produced. It is possible that seed production has been overestimated, for example because pollination may have been limiting, due to a lack of pollinators or poor weather conditions. The density of plants in the mechanically harvested field is also lower than in the manually harvested field, but the relative areas of these two fields are not known. As a conservative estimate, at least 100 million TKS seeds escaped from the nurseries at Olsgården between 1947 and 1950.

Dandelion seeds can be spread over great distances by wind. Using the dispersal distributions obtained in the computer simulations by Tackenberg et al. (2003, see the introduction), this equals 60,000 seeds dispersing more than 100 m and 14,500 seeds more than 1 km. However, long-distance dispersal is typically erratic, and some seeds may disperse much further.

Escaped plants, after dispersal, can become a secondary source for new dispersal. Although TKS is self-incompatible, this system is leaky, especially at the end of the flowering season (Warmke 1944), and self-fertilization can also occur through mentor pollen effects caused by pollen from triploid dandelions, as mentioned in the introduction. Therefore, a few escaped TKS plants can start a new wild population and become a new secondary gene flow source. The greatest chance of finding invaded TKS is a targeted search in the area of the former seed production sites, as was done in this study. However, TKS plants were not observed. The present authors obtained TKS hybrids in many interspecific (back)crosses, and J. Kirschner is a *Taraxacum* taxonomist and an expert of the *Taraxacum* section *Ceratoidea* to which TKS belongs. Therefore, it is unlikely that pure TKS or early BC plants were overlooked at the sites where the chance of finding them was greatest. It may be argued that TKS could be found at sites not investigated, but despite being morphologically very distinct from native dandelion species, TKS is not reported as a component of the Swedish flora (Karlsson & Agestam 2019, Kirschner et al. 2007+). It seems that after the termination of the TKS growing program in 1952 in southern Sweden, TKS soon became extinct. Cultivations of TKS in the USA during WWII suffered severely from the competition of weedy dandelions, reducing the root harvests significantly (Whaley and Bowen 1947). In general, TKS is a small plant, and poor performance of TKS was observed in competition experiments in Germany (Uhlemann et al. 2019).

So far, this discussion has been about pure TKS plants. We estimated that 125 million TKS ovules could be fertilized each year in Olsgården by TKS or TO pollen. Artificial crosses have shown that hybrids can be produced by the pollination of TKS by triploid apomicts (Iaffaldano et al., 2018). This will be the most important route of gene flow, but there is (theoretically) the possibility that sexual diploid TO is fertilized by TKS pollen. Sørensen (1958) documented experimentally that a sexual diploid *T. officinale s. lat.* can derive at very low frequencies from apomicts by autosegregation. Gustafsson (1937) found *Taraxacum obtusilobum* Dahlst. from Göteborg, W. Sweden, sexual and diploid, occasionally forming hybrids with triploids surrounding it in the wild. Alternatively, there is the possibility that apomixis in polyploid TO is facultative (apomicts that produce a part of apomictic and another part of sexual egg cells, see also Sørensen & Guđjónsson 1946, Sørensen 1958), and those sexual ovules are fertilized, possibly by TKS pollen. However, Mártonfiova *et al.* (2006, 2010, 2015) could not find evidence for facultative apomixis in TO nor in the *T.* sect. *Erythrosperma*.

A part of the experimental hybrids made by cross-pollination of TKS by pollen of *T. officinale* was apomictic (Iaffaldano et al. 2018). This is consistent with the finding that elements of apomixis (apomeiosis and parthenogenesis) in *T. officinale* are controlled by dominant major genes (Mártonfiová 2006). Since the elements of apomixis are not genetically linked, only about one-quarter of the hybrids will be fully apomictic (van Dijk et al. 2020). These autonomous apomictic hybrids produce autonomous endosperm and can propagate without partners and establish a new population by themselves. The hybridogenous origin of apomictic plants may not be evident from morphology because of TKS traits' recessivity or repeated backcrosses. Iaffaldano et al. (2018) found that TO traits in TKS x TO hybrids were dominant.

Given these considerations, we carried out a population genetic study of dandelion populations at 21 locations varying in distance to the original seed nurseries. Most samples could be reliably scored with the codominant markers. KASP™ set I showed a higher rate of dropouts than set II, probably because set II consisted of the six most robust gene markers out of the original number of 13 that were developed, whereas set I was restricted to a few genes with a specific function. Still, a large number of plants could be genotyped for the rubber biosynthesis genes. For nine out of ten markers, wild dandelions were fixed for the non-TKS marker. For the tenth marker, the wild dandelions were fixed heterozygous. This 10th marker is discussed below. Based on the assumption of triploidy, around 14,500 alleles were screened with the nine codominant markers. None of these alleles was a TKS-specific allele in the populations within 2.5 km of the former seed production areas. This is consistent with the absence of TKS morphological characteristics at the sampled locations. It can be concluded that introgression did not occur or is so rare that it is not detectable in a large sample.

There are several possible explanations why introgression in Sweden is minimal. Mártonfiová (2006, 2015) and Mártonfiová et al. (2010) found that in *T. officinale* in mixtures of pollen from diploid and triploid plants, the triploids were not effective fertilizers. Another factor may be that intra-specific pollen from TKS fertilizes better than inter-specific pollen from TO. Furthermore, the establishment can be an important introgression limiting factor because new apomicts have to compete with established

apomictic clones. Although Iaffaldano et al. (2018) found that TO traits in TKS x TO hybrids were dominant, hybrids seemed to be less adapted than pure TO plants. The results of the present study (a lack of invasiveness and introgression) may be extrapolated to future TKS cultivation in regions where only apomictic dandelions grow, such as Europe north of 50° latitude or in North America (van Dijk 2003).

Marker Contig2608-372 behaved differently from the other markers in the sense that this marker was fixed heterozygous. If this were due to the introgression of TKS genes, it would be expected that the highest levels of TKS introgression would be found close to the seed production sites. However, this is not the case because even populations A and U at distances of 12.3 km and 25 km were fixed heterozygous. The likely explanation is that this marker is genetically linked to an apomixis locus, which is also fixed heterozygous (Aaa). This has been found for a Simple Sequence Repeat (microsatellite) marker in a Danish population of *T. officinale* (van der Hulst et al. 2003), of which it was known from experimental crossings that this genetic marker was linked to the diplospory apomixis locus(van Dijk and Schotman 2004; van Dijk et al. 2009). Van Dijk et al. (2020) describe another SSR marker that is linked to the parthenogenesis locus in *T. officinale*. Consistent with the idea of linkage to an apomixis gene is the fact that the gene was homozygous in the diploid sexual TO test population. Although the marker Contig2608-372 has not been genetically mapped, the fact that the the different Kazakhstan apomicts, which belong to different *Taraxacum* species, were also all heterozygous for this locus (Table 5), supports the idea that this locus is genetically closely linked to a functional apomixis gene.

Conclusions

In the regions with natural occurrence of TKS occasional hybridization with other dandelions occasionally takes place in the historical perspective (and polyploid apomicts of such parentage exist). The present screening of mixed TKS populations did not reveal any traces of introgression.

The screening of areas of TKS cultivation in Sweden yielded similar results. There is a negligible risk of introgression from TKS to other *Taraxacum* species in the regions without other sexual dandelions or at least without related dandelions.

Declarations

Conflict of interest

The authors declare that there is no conflict of interest.

Author Contribution

P.J. v.D. designed the subject and methods and prepared the first version of the manuscript. Judith K., R.A.M. and P.J. v.D. performed the laboratory work and cultivation experiments. Jan K. and J. Š. collected the material (in Sweden, together with R.A.M. and P.J. v.D.) and provided the taxonomic background for the analysis of material. Jan K. and P.J. v.D. edited subsequent versions of the Ms. and prepared it for submission.

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Data Availability

All data supporting the findings of this study are available within the paper. Raw data for the KASP analysis are available upon request .

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Tables

Tables 2, 4 & 5 are available in the Supplementary Files section.

Figures

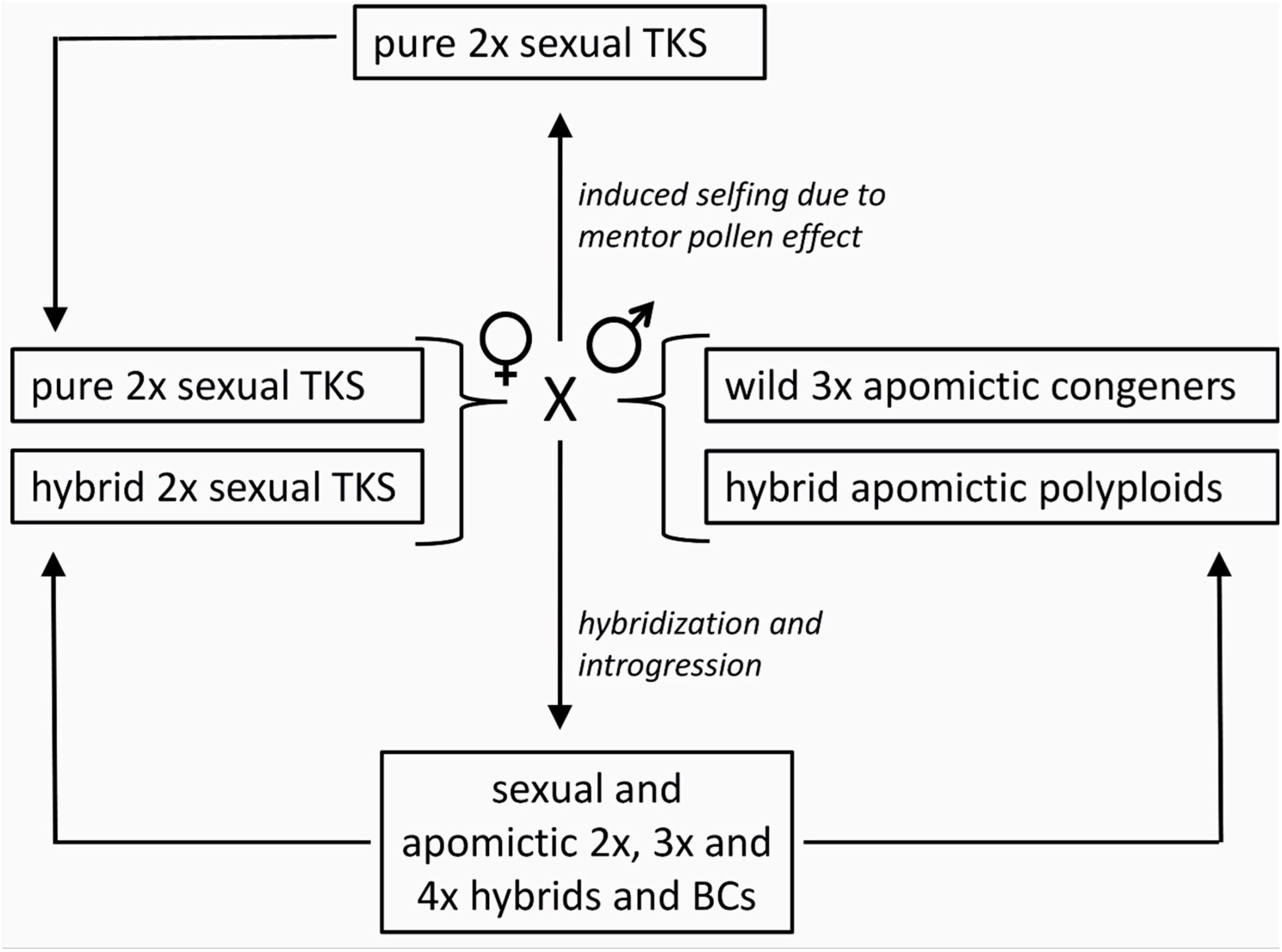


Figure 1

Potential routes of introgression between TKS and congeners (lower part) and induced selfing due to breakdown of the Self Incompatibility system by the pollen mentor effect. BC means back cross.





Figure 2

Temporarily wet pastures with springs, locally with dense stands of TKS. Tuzkol Lake shores, southeastern Kazakhstan.

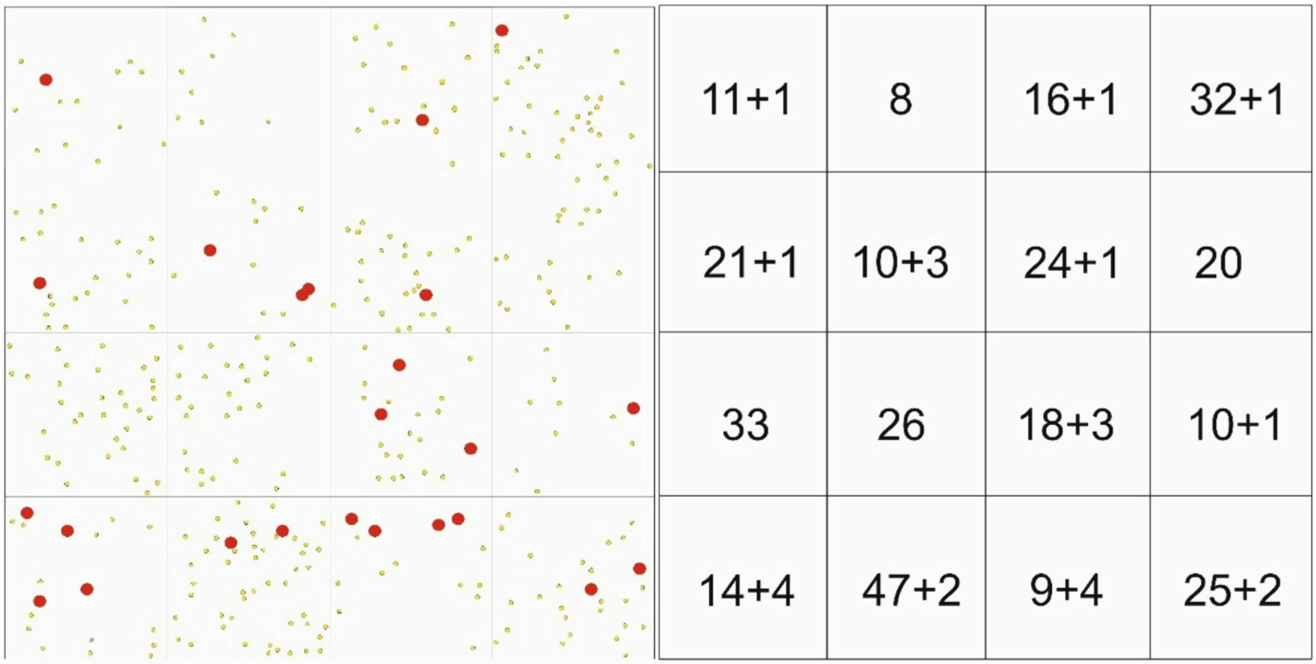


Figure 3

Example of the coexistence of TKS and other dandelions. The habitat is seminatural, with excessive grazing and other types of disturbance. The picture shows one of the permanent plots established for monitoring the TKS populations near Saryzhaz in 2014. It is selected for a relatively high number of non-TKS dandelions. Left: A graphical record of TKS (little yellow dots) and other dandelions (bigger red dots). Right: The census of TKS + other dandelions on the plot. Permanent plot II (4 × 4 m), 324 plants of TKS, 24 other dandelions. Identification codes of 1 m^2 squares within the plot: 168–183. Coordinates 42°55.770'N, 79°40.161'E, 1892 m a.s.l.

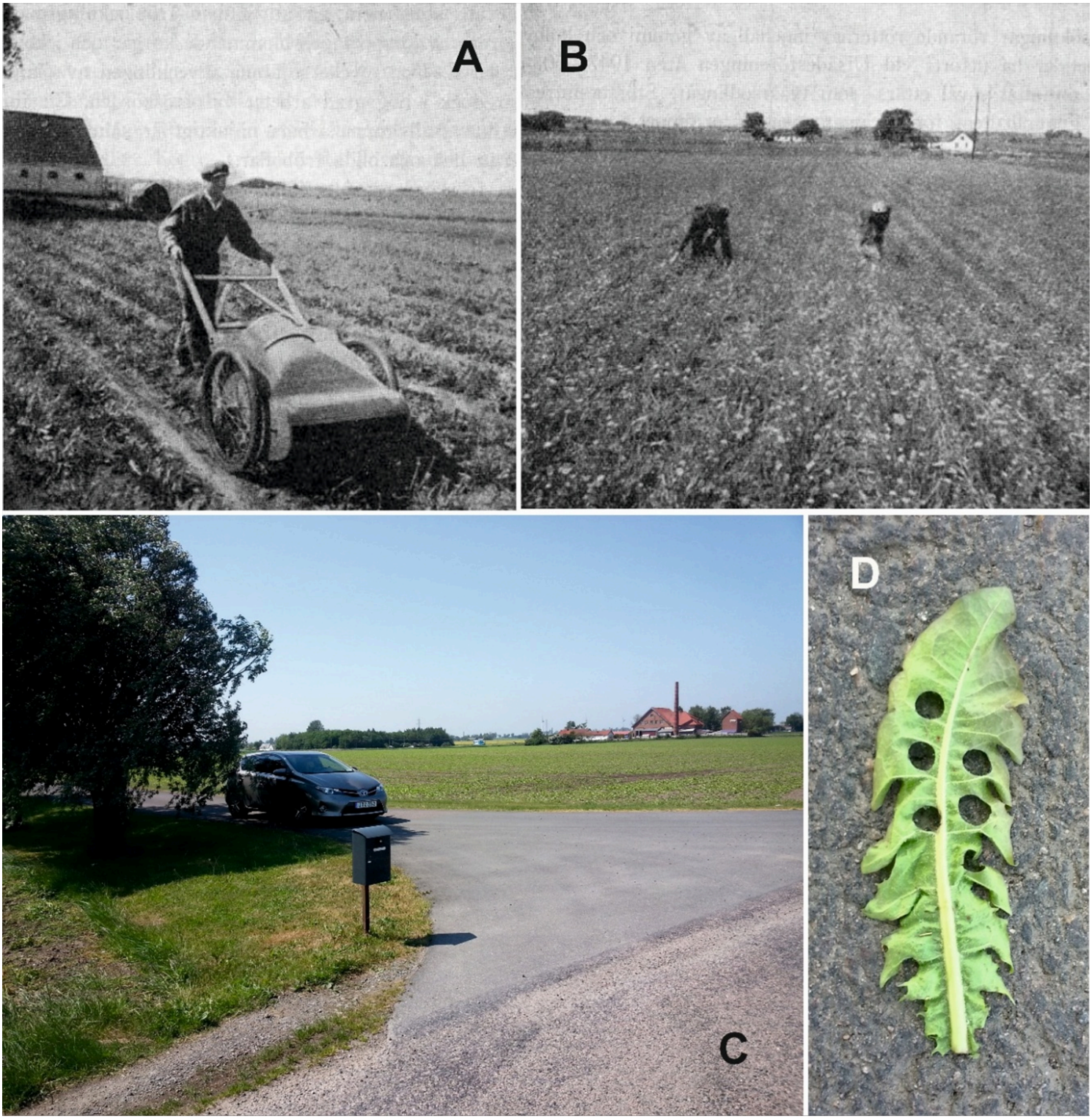


Figure 4

A, growing TKS for seed production at the Tomelilla agricultural school (Josefsson 1953). **B**, TKS seed harvest at the Olsgården farm Hammenhög (Josefsson 1953). **C**, The entrance to the Olsgården farm in 2016. Dandelions were sampled from the road verge. **D**, the technique of sampling.

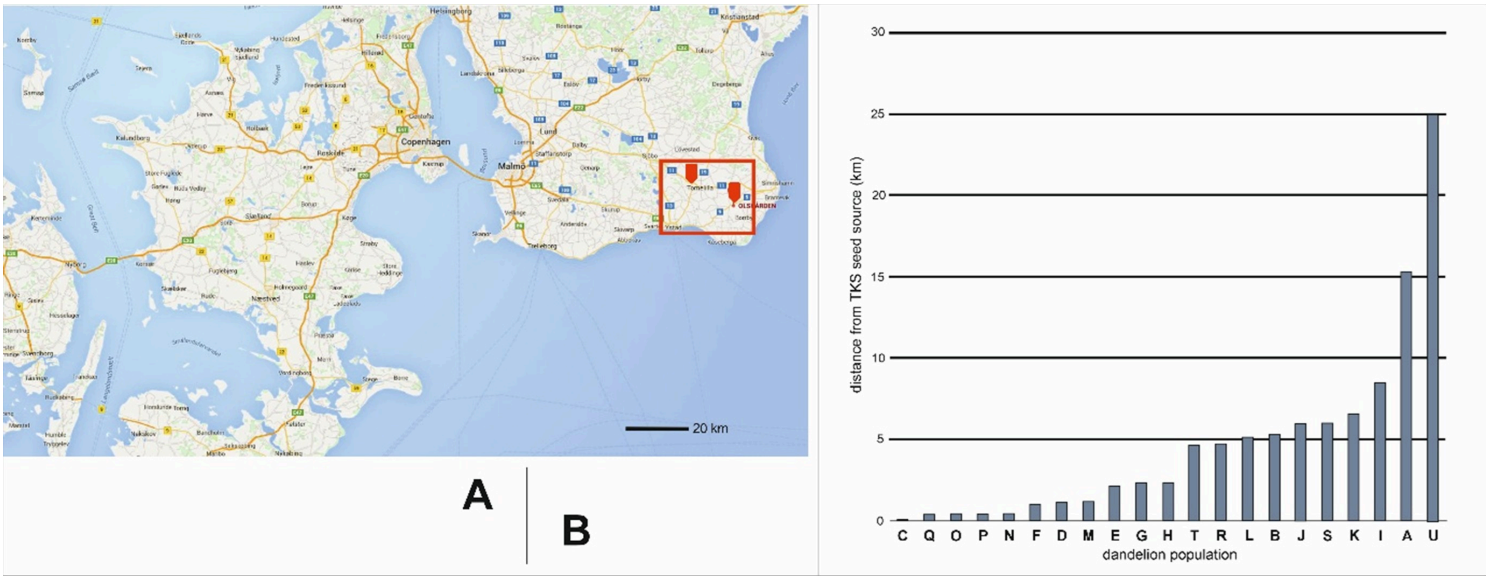


Figure 5

A, The location of the sampling area in Skåne, southern Sweden. **B,** The distribution of the distances of the sampling localities from the nearest former seed nursery.

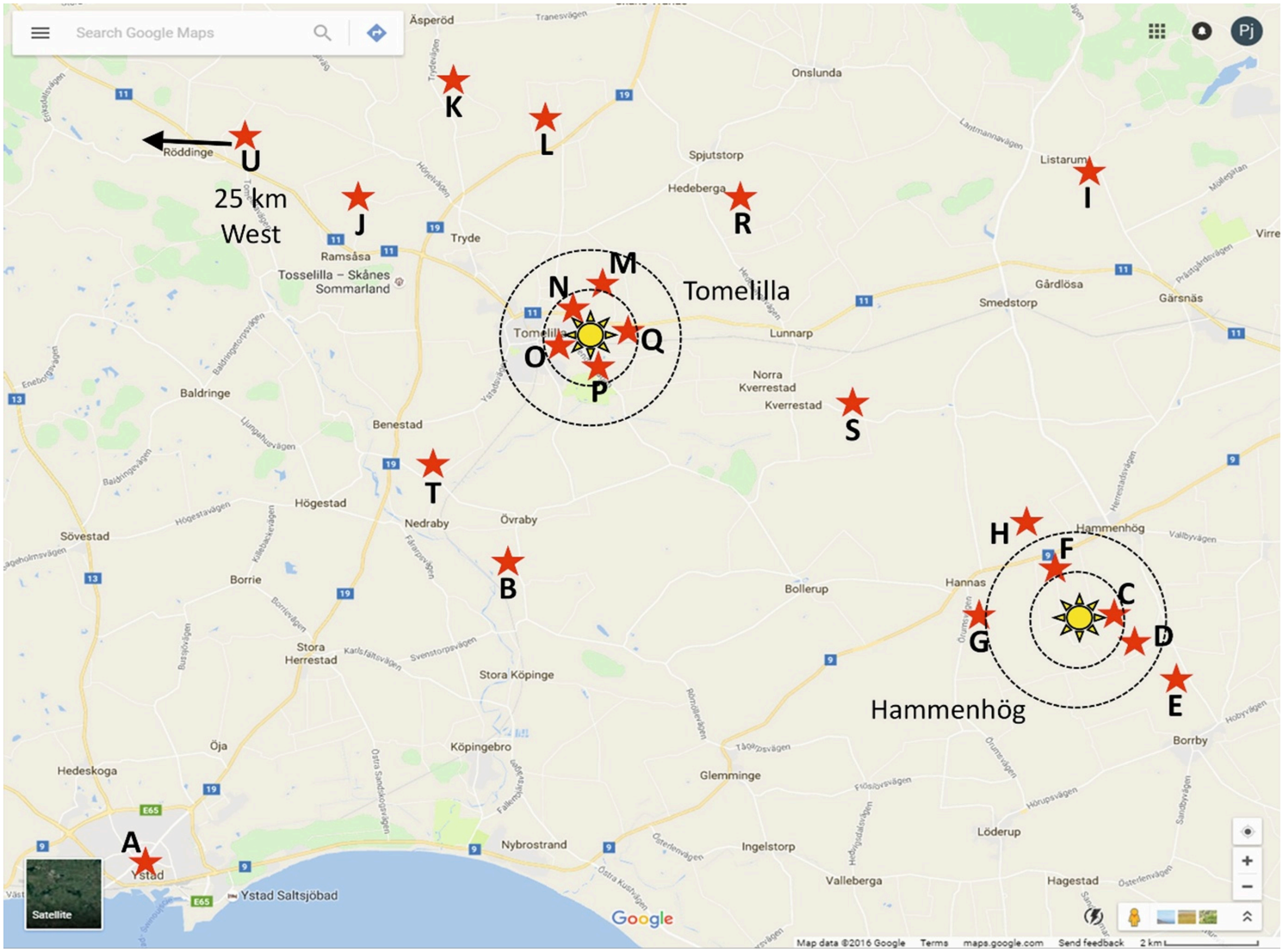


Figure 6

A map of the sampling area in Skåne with the locations of the sampled dandelion populations. The dashed circles indicate a circle with radius of 1 and 2 kilometer, respectively around the two historical seed nurseries, Tomelilla and Hammenhög.

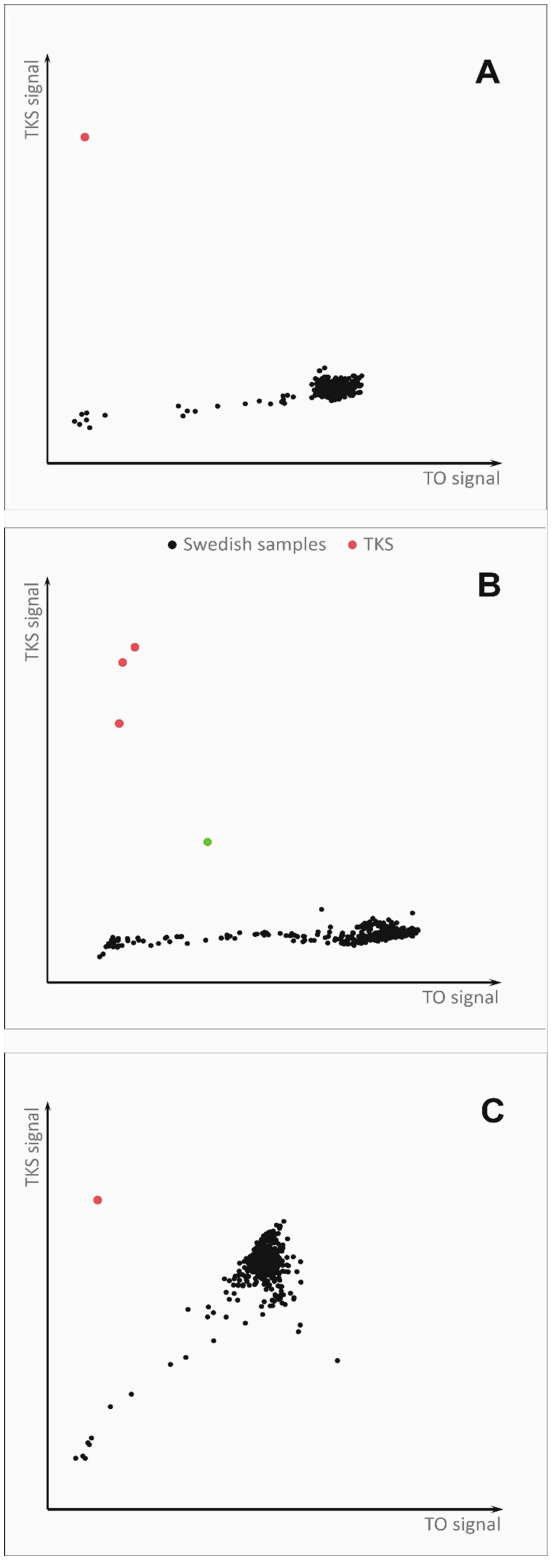


Figure 7

Different types of KASP biplots. The fluorescent signal for the two wavelengths is plotted for each sample and for control samples. **A**. The allelic biplot for SNP Contig179-397, a typical biplot for eight assays. All samples collocate with the pure TO sample (black) and none with the pure TKS sample (red). **B**. The allelic biplot for SNP Contig 2608-372. All except one sample collocate with the TO sample (black), however, there is one sample with a strong signal for both alleles (green). **C**. The allelic biplot for

SNP Contig 2608-372, showing fixed heterozygosity. Dots close to the interception of ordinate and abscissa represent poor amplification (drop out).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table245.docx