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Search for evidence of introgression of wheat (*Triticum aestivum* L.) traits into sea barley (*Hordeum marinum s.str.* Huds.) and bearded wheatgrass (*Elymus caninus* L.) in central and northern Europe, using isozymes, RAPD and microsatellite markers

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Abstract Seeds of English and Austrian populations of bearded wheatgrass (*Elymus caninus* L.) and sea barley (*Hordeum marinum* Huds.) growing in the vicinity of wheat (*Triticum aestivum* L.) fields were collected in order to search for evidence of the introgression of wheat traits into these wild relatives. Seeds were sown and plants grown for subsequent analyses using morphological and genetic (isozymes, RAPD and wheat microsatellites) markers. No F_1 hybrids were found within the individuals of the two species grown, neither with morphological nor with genetic markers. Also, no evidence of introgression of wheat traits into *E. caninus* was observed. However, in one individual of *H. marinum* which had the typical morphology of this species, numerous species-specific DNA markers of wheat were amplified, thereby demonstrating previous hybridization. Consequently, the hybridization between wheat and *H. marinum* under natural conditions and the introgression of wheat traits into this wild relative seems to be possible. Our results contribute to the risk assessment of transgenic wheat cultivation.

Keywords Wheat · Wild relative · Gene flow · Introgression · Genetic marker

Introduction

Among the concerns related to genetic engineered crops, the risk of gene escape toward wild flora is one of the most discussed. The potential for such gene flow is directly proportional to the potential of crop-wild hybridization (Ellstrand and Hoffman 1990). For numerous crops, wild relatives are known that can hybridize with them somewhere in the world. This is the case, among others, of maize (Doebley 1990), oilseed rape (Klinger et al. 1992), sunflower (Whitton et al. 1997) and sugar beet (Bartsch and Pohl-Orf 1996).

For wheat, the center of distribution is assumed to be the Middle East and the Mediterranean area. Many wild species closely related to wheat exist in these regions; most belong to genus *Aegilops* and grow close or within wheat fields, frequently hybridizing with this crop (van Slageren 1994). More generally, the *Triticeae* tribe is distributed worldwide throughout the temperate regions of both hemispheres (Miller 1987). Many wild relatives of wheat have been introduced as adventive in other regions and even on other continents, as is the case for *Ae. cylindrica* in North America (Donald and Ogg 1991).

In northern Europe two wild relatives of wheat, sea barley (*Hordeum marinum* Huds.) and bearded wheatgrass (*Elymus caninus* L.), occasionally grow in direct proximity or even within wheat fields and have been described as being not strictly autogamous (Sun et al. 1997; De Bustos et al 1998). Several studies have described the production of viable and partially fertile hybrids between these wild species and wheat for agronomic purposes (Sharma and Baezinger 1986; Fedak 1991). Considering their breeding system, their belonging to the same tribe and the fact that they often grow within a huge amount of wheat pollen, the risk of spontaneous hybridization with wheat cannot be excluded.

In order to estimate the ecological risks involved with field trials or wide cultivation of transgenic crops, it is essential to determine the potential for such hybridizations to occur under natural conditions. A compilation of the literature shows that there is no evidence that the pollen production of transgenic and conventional crops differs essentially. Therefore, the search for hybrids between conventional wheat cultivars and wild relatives will be a good measure of the risk of gene escape from

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transgenic wheat. Indeed, any hybridization will depend on the outcrossing rate of the recipient species rather than on the pollen production of the crop. This kind of study can be considered as a necessary step prior to field tests with genetically engineered crops.

The aim of the study presented here was to estimate the potential of spontaneous hybridization between wheat and two wild relatives, *Hordeum marinum* s.str. Huds (*H. marinum* subsp. *marinum*) and *E. caninus* L. and to search for evidence of the introgression of wheat DNA into these wild species. Morphological, DNA [randomly amplified polymorphic DNA (RAPD) and microsatellite] and enzyme markers were used for this purpose.

Materials and methods

Plant material

Seeds of the two wild species were collected in Austria and England (Fig. 1, Table 1). In each sampled population (Table 2), a representative sample of 20–40 spikes (1 spike/plant) was collected. Two populations of *Hordeum marinum* subsp. *marinum* (England) and six populations of *Elymus caninus* (England and Austria) were sampled.

Wheat (*Triticum æstivum*) seeds were collected in the immediate proximity of the wild relatives populations in order to obtain specific markers and detect their possible introgression into wild genomes. In addition, we included in the study five Swiss wheat varieties (seeds obtained from Eric Schweizer Samen AG, (Thun). Seeds were sown and plants cultivated in the Botanical Garden of Neuchâtel.

Morphological markers

Specific morphological markers were determined on pure plants of each species (Table 3) and used to analyze the offspring of the sampled populations.

Genetic markers

Specific genetic markers, obtained with isozymes, RAPD and microsatellite techniques, were set up as described in a previous paper (Guadagnuolo et al. 2001a) and used to analyze the offspring of the sampled populations. Two enzyme systems, glutamate oxaloacetate transaminase (got) and peroxidase (prx), eight RAPD primers (OPB 6, 8, 10 and OPP 6, 7, 8, 9, 14; Operon Technologies, Alameda, Cal.) and six publicly available wheat microsatellite primers pairs (WMS 43, 44, 46, 47, 106 and 159; Plaschke et al. 1995; Röder et al. 1995) were used as they produced species-specific markers. The amplified microsatellites were described being present in a specific genome of *T. æstivum* (Plaschke et al. 1995; Röder et al 1995). However, some of them amplified specific bands in the wild relatives, as already demonstrated by Sun et al. (1997) for *Elymus* species.

Isozyme analyses

Protein extraction from 451 *E. caninus*, 88 *H. marinum* and 35 *T. æstivum* plants (Table 2) was carried out by grinding two young leaves per plant in 1 ml 0.1 *M* sodium acetate solution (pH 7.2). The extracts were then centrifuged at 12000 rpm and the supernatant stored at –80 °C for subsequent isozymes analyses.

Polyacrylamide gel electrophoreses (PAGE) (2.5 mm thick) were prepared according to Gasquez and Compoint (1976), using the modifications of Lumaret (1981): sample gel (9% acrylamide and 0.165% bis-acrylamide); stacking gel (2.5% acrylamide, 1 cm long) and separation gel (9% acrylamide and 0.165% bis-acryl-

Table 1 The species studied and their genetic characteristics (Miller 1987; Sun et al. 1997; Baum and Johnson 1998)

Species	Genome formula	Ploidy level
Triticum æstivum L. (common or bread wheat)	AABBDD	2n=6X=42
Elymus caninus L. (bearded wheatgrass or b. couch)	SSHH	2n=4X=28
Hordeum marinum Hudson *s.str.* (sea barley)	XX	2n=2X=14

Table 2 Sampling of the species and of the individuals for genetic analyses

Species	Populations/varieties	Identification names (Fig 1)	Number of plants analyzed with DNA markers	Number of plants analyzed with morphological and enzyme markers
E. caninus	England (GB) and Austria (Aut)			
	Warboys Wood Nature reserve (2 subpops) (52°25′N; 0°05′W)	Ec.2GB	10	26
	Horncastle (53°9′N; 0°08′W)	Ec.6GB	5	54
	Scottlehorpe (52°46′N; 0°26′W)	Ec.7GB	5	46
	Sonthey Wood, Peterborough (52°35′N; 0°22′W)	Ec.8GB	5	93
	Achleiten (2 subpops) (48°5′N; 14°11′E)	Ec.1Aut	10	133
	Kaltenbach (2 subpops) (48°16′N; 13°53′E)	Ec.2Aut	10	97
H. marinum	England (GB)			
	Wolferton, Norfolk (2 subpops) (52°51′N; 0°27′E)	Hm.3GB	10	65
	Sonthey Wood, Peterborough (52°35′N; 0°22′W)	Hm.9GB	5	23
T. æstivum	1 Austrian variety (Favorit)	Tae.Aut.var	1 bulk of 10	10
	3 English varieties (unknown)	Tae.GB1–3	3 bulks of 10	30
	5 Swiss varieties (Arina, Galaxie, Tamaro, Boval, Runal)	Tae.CH.var	5 bulks of 10	50

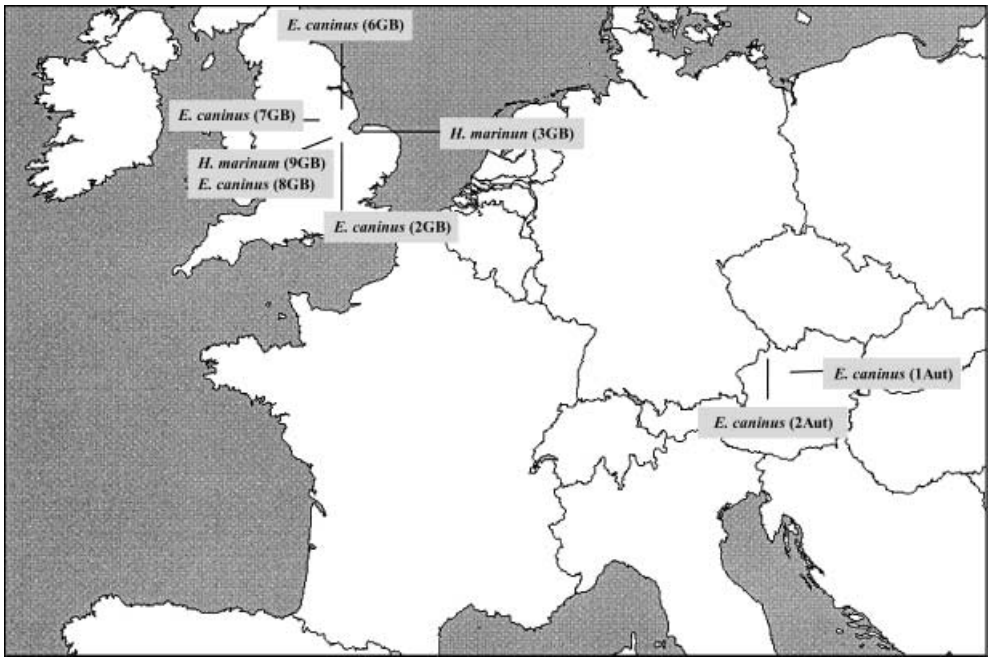

Fig. 1 Sampling sites of the wild relatives of wheat (identification numbers of populations in *brackets*)

Table 3 Main morphological characteristics of the species studied

Traits	*Triticum aestivum*	*Elymus caninus*	*Hordeum marinum*
Culm	100–150 cm	30–110 cm	10–40 cm
Leaves	Long, broad	Shorter, smaller, bluish-green	Leaf blade sometimes rolled up, inside with few hairs
Spike	Thick, compact; whitish, yellow, reddish color	Thin, drooping to one side	Compact, short
Spikelets	2 to 4 in a row, thick	Mostly 3 in a row, thin	Always 3, middle spikelet fertile, side spikelets sterile
Awns	Depends on varieties	Awns longer than glumes, wavy	Glumes of side and middle spikelets awned, palea of middle spikelet awned

amide, 7.5 cm long). Forty microliters of each sample (mixed with 20 µl of dye bromophenol blue) migrated in a TRIS-glycine (pH 8.6) buffer at 4°C under the following conditions: 10 min at 600 V and 6 pps (pulsations/s), 20 min at 230 V and 7 pps and 2.5 h at 600 V and 7 pps.

Protein separation was performed within 2 days after the extraction. Enzyme staining was carried out as described in Savova Bianchi (1996).

DNA analyses

Total DNA extraction from a single leaf was carried out on five samples per population (or subpopulation) for *H. marinum* and *A. caninum*. For each of the wheat varieties, the extraction was performed on a bulk of leaves of ten plants using a simple SDS-na-acetate protocol (Savova Bianchi 1996), the DNA was resuspended in TE (TRIS-EDTA, pH 8) at a concentration of 30 ng/ml and stored at −20°C. In total, 15 *H. marinum*, 45 *E. caninus* and nine *T. æstivum* DNA samples were analyzed.

Polymerase chain reactions (PCR) for both RAPD and microsatellite amplifications were performed in a volume of 25µl under the following final conditions:

RAPD

The reaction volume consisted of 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.4× Q-solution (Qiagen AG, Basel), 0.2 m*M* dNTP, 0.2 µ*M* primer, 0.03 U/µl *Taq*-pol (Qiagen AG, Basel) and 1 ng/µl template DNA. Amplifications were performed in a Biometra I thermocycler using the following profile: initial denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 93°C, 1 min at 45°C and 1 min at 72°C; a final extension was for 10 min at 72°C. PCR products were mixed with 1/5 vol loading buffer and separated on a 1.6% (w/v) agarose gel containing 0.4 µg/ml ethidium bromide in 0.5× TBE at 60 V for 2 h. The DNA fragment were then visualized under UV light.

Microsatellites

The reaction volume consisted of 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.4× Q-sol. (Qiagen AG, Basel), 0.2 m*M* dNTP, 0.6 n*M* each primer, 0.03 U/µl *Taq*-pol (Qiagen AG, Basel) and 1 ng/µl template DNA. Amplifications were performed in a Biometra I thermocycler using the following profile: initial denaturation at 93°C for 3 min, followed by 45 cycles of 1 min at 93°C, 1 min at 55°C and 2 min at 72°C; a final extension was for 10 min at 72°C. PCR

products were mixed with 1/5 vol loading buffer and separated on a 6% polyacrylamide gel in 0.5× TBE at 100 V for 6 h. Gels were stained in a 0.4 µ g/ml ethidium bromide solution, and DNA fragments were vizualized under UV light.

Data analysis

The data obtained with the three techniques were scored in a binary form as the presence or absence (1 and 0) of bands for each individual or bulk (in the case of wheat DNA samples). We used the R4 (Beta version) package (Philippe Casgrain and Pierre Legendre, Université de Montréal) to calculate Jaccard's similarity coefficient and, after conversion of the similarity matrices into distance matrices, to perform Principal Coordinates Analyses (PCoA).

Results

Elymus caninus

None of the 450 offsprings showed intermediate morphology between the two parental species and detailed morphological analyses (data not shown) confirmed general observations. In addition, none of the specific DNA or enzyme markers of wheat was found in any of the progeny of wild plants. Therefore, no evidence of introgression of wheat DNA into bearded wheatgrass was found.

Principal Coordinates Analyses performed on the distance matrices obtained with the three different types of markers confirmed these observations. Indeed, in all cases, wheat and *E. caninus* individuals grouped separately (e.g. Fig. 2).

Hordeum marinum

In *H. marinum*, indications of hybridization with wheat were found in the analyzed plants. None of these individuals had a morphological appearence intermediate between those of wheat and sea barley (data not shown). However, despite its typical *H. marinum* morphology, one offspring of the population of Sonthey Wood (H.m. 9GB-130, Fig. 1) amplified one out of eight microsatellites (Fig. 3) and 8 out of 32 RAPDs specific markers of wheat, while it exhibited none of the enzyme markers of wheat. These nine DNA fragments were also absent in all *E. caninus* individuals, including those harvested 5 m away from this sample (E.c. 8 GB, Fig. 1). None of the other *H. marinum* individuals possessed wheat DNA- or enzyme-specific markers.

PCoA performed on the isozyme based distance matrix showed that all the *H. marinum* samples were well separated from wheat. The samples of the two species

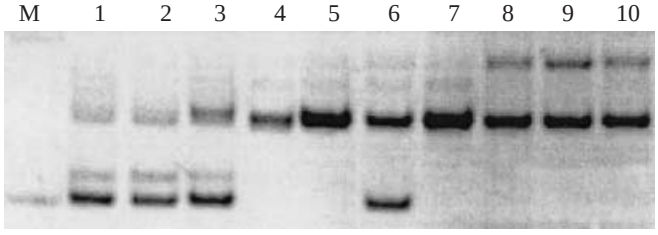

Fig. 3 Example of microsatellite amplification in *T. æstivum* (*lanes 1–3*) and *Hordeum marinum* (*lanes 4–10*) with primer WMS-46. *M* 100-bp DNA ladder (GibcoBRL, Life Technologies), showing one fragment specific for wheat present in one individual of *H. marinum* (H.m. 9GB-130)

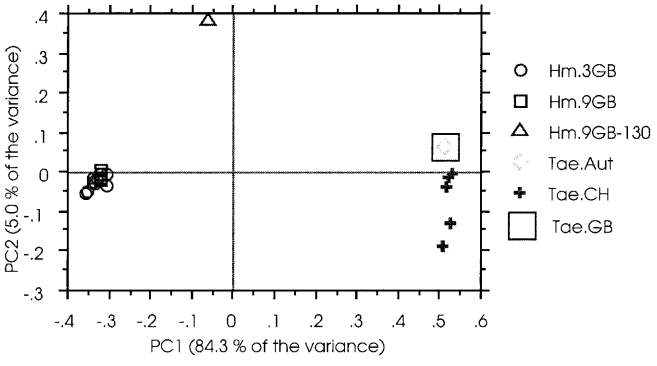

Fig. 4 Principal Coordinates Analysis (PCoA) based on RAPDs data (Jaccard's similarity coefficient), *Hm H. marinum*, *Tae* T. *æstivum*

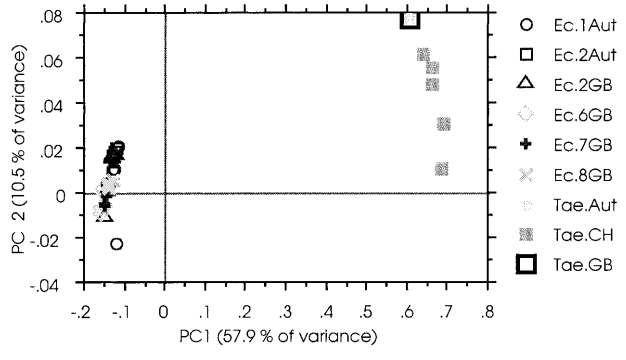

Fig. 2 Principal Coordinates Analysis (PcoA) based on RAPDs data (Jaccard's similarity coefficient). *Ec Elymus caninus*, *Tae Triticum æstivum*

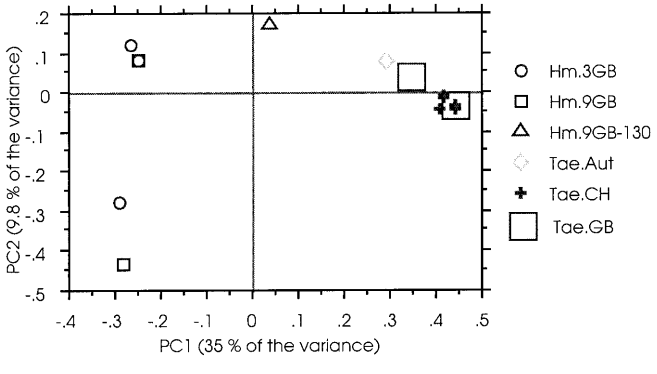

Fig. 5 Principal Coordinates Analysis (PCoA) based on microsatellites data (Jaccard's similarity coefficient), *Hm H. marinum*, *Tae T. æstivum*

were also well separated by the analyses performed on the RAPD and microsatellite distance matrices, with the exception of the individual that amplified specific DNA markers of wheat, which had an intermediate position (Figs. 4 and 5).

Discussion

Hybridization

Spontaneous crop-wild relative hybridization has been shown to be more frequent than previously expected (e.g. Ellstrand et al. 1996). However, because most hybrids are almost completely sterile or the inherited crop traits are maladaptive, conventional crop traits generally do not persist in wild populations. On the other hand, the persistence of foreign traits in one species is possible if hybrids can backcross with one of the parental species and produce viable seeds (Zemetra et al. 1998).

Data obtained with all different types of markers and based on the analysis of 450 individuals showed no evidence of the introgression of wheat traits into *E. caninus* growing within or in the direct proximity of wheat fields. With bearded wheatgrass, even a single hybridization event could have long-term effects. Indeed, the likely partial sterility of such a hybrid would be counterbalanced by its perennial habit, allowing several years to obtain some reproductive success (Ellstrand et al. 1996).

On the contrary, in one sample of *H. marinum* numerous DNA-specific markers of wheat were amplified, that were absent in all the other samples of the same species, including the progeny of population H.m. 3 GB (Wolferton) collected 20–50 m away from the cultivation area. A trait shared by a crop and a wild relative can be the result of either hybridization or the inheritance from a common ancestor (Doebley 1990). However, the presence of crop traits in a population of a wild species growing in agroecosystems, which are absent in populations of the same species isolated from the influence of agriculture, will be an indication of introgression. Because such hybridizations are generally rare events, the presence of only few individuals that possess crop traits within a population of a wild species also indicates introgression. Our results could indicate that at least one hybridization event between the two species has occurred in previous generations. Subsequent backcrosses of the hybrid with pure *H. marinum* could then have led to the introgression of wheat DNA into sea barley. Notwithstanding this species was previously considered to be essentially autogamous, our findings indicate that outbreeding cannot be excluded. This confirms recent results obtained by De Bustos et al. (1998) describing at least partial outbreeding in Spanish populations of the same species.

Whether if the putative hybridization described above is a recent or ancient event can not be determined by only looking at the morphological appearance. Indeed, in a previous study (Guadagnuolo et al. 2000b) we observed that BC_1 plants resulting from backcrosses between *Aegilops cylindrica*×wheat hybrids, and *Ae. cylindrica* had the same morphology as the latter. This indicates that probably one or two backcrosses are sufficient to restore the main morphological traits.

Only DNA markers detected crop traits in the sample H.m. 9GB-130, while the expression of two enzyme systems was identical to that of the other sea barley individuals. Isozymes reflect variation only in the coding parts of the genome, which are probably eliminated during the introgression process if they do not confer adaptative advantages. On the contrary, non-coding DNA (amplified with RAPD and microsatellite primers) can be integrated without affecting the fitness of a plant and thus persist more easily.

Relevance for risk assessment of transgenic wheat cultivation

There is general agreement for considering hybridization between crops and wild relatives as a danger for biodiversity. However, traits introduced in crops by conventional breeding are generally already present in wild plants. Therefore, the consequences of eventual crop-to-wild gene flow should be minor. Moreover, the traits commonly selected in conventional – *i.e.* non-transgenic – crops, like dwarfing or absence of dormancy, do not represent competitive advantages for a wild species (Ellstrand and Hoffman 1990). Even if hybridization occurs, the persistence of this kind of crop gene in the wild is hence unlikely. In addition, because crops and wild relatives are often of different ploidy levels, putative hybrids are mostly sterile and generally eliminated by selection. It is consequently difficult to detect evidence of past introgressions of conventional crop traits into wild species if they do not confer ecological advantages.

The absence of hybrids and introgressants in the progeny of *E. caninus* growing close to wheat fields indicates a very low risk of crop-to-wild gene flow. Therefore, gene escape from transgenic wheat to this wild relative seems unlikely. On the contrary, relatively strong indications of the introgression of wheat DNA into *H. marinum* growing on the border of wheat fields was found. Sea barley generally grows at seasides or inland on saline soil, and its presence in the vicinity of areas of wheat cultivation is not frequent. In order to avoid gene escape from transgenic wheat fields, an isolation distance is thus required, the extent of which will depend on the distance that wheat pollen can cover but also on its longevity. We have found no report in the literature in which the pollination of wild species by wheat was investigated as a function of distance. Several studies, however, have estimated the maximum distance for effective cross pollination (*i.e.* resulting in seed set) between wheat cultivars (de Vries 1974; Kertesz et al. 1995). The results of these studies indicated that cross fertilization decreased to 10% at 3 m and was just detectable at 20 m from the source. Moreover, repeated polli-

nation events were necessary to activate the pollen growth on the stigma (de Vries 1974; Fritz and Lukaszewski 1989), which indicates that few pollen grains will not necessarily fertilize the recipient species. It is thus likely that, independently of the target species, maximum pollination distance will be a few tens of meters. In addition, Poaceae pollen is generally short lived (Zandonella 1984). Fritz and Lukaszewski (1989) found a maximum longevity of 1 hour for wheat (cv. Chinese Spring) pollen, which could decrease severely in the case of hot and dry conditions, as is the case with strong wind in summer. Therefore, an isolation distance of 20–30 m should avoid or strongly reduce the risk of gene escape from transgenic wheat fields to sea barley.

Conclusion

Spontaneous hybridization between wheat and *E. caninus* has not been shown and, thus, introgression of wheat traits into this wild species seems unlikely, regardless of the fact that it often grows in the direct vicinity of wheat fields.

Despite limited contact zones and distant phylogenetic relationships, we found evidence that wheat traits can be transferred into *H. marinum*. As a precautionary principle, an isolation distance of at least 20–30 meters should be required to avoid the risk of gene escape from transgenic wheat fields to sea barley.

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