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The Red Queen and the seed bank: pathogen resistance of *ex situ* and *in situ* conserved barley

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Abstract

Plant geneticists have proposed that the dynamic conservation of crop plants in farm environments (*in situ* conservation) is complementary to static conservation in seed banks (*ex situ* conservation) because it may help to ensure adaptation to changing conditions. Here, we test whether collections of a traditional variety of Moroccan barley (*Hordeum vulgare* ssp. *vulgare*) conserved *ex situ* showed differences in qualitative and quantitative resistance to the endemic fungal pathogen, *Blumeria graminis* f.sp. *hordei*, compared to collections that were continuously cultivated *in situ*. In detached-leaf assays for qualitative resistance, there were some significant differences between *in situ* and *ex situ* conserved collections from the same localities. Some *ex situ* conserved collections showed lower resistance levels, while others showed higher resistance levels than their *in situ* conserved counterparts. In field trials for quantitative resistance, similar results were observed, with the highest resistance observed *in situ*. Overall, this study identifies some cases where the Red Queen appears to drive the evolution of increased resistance *in situ*. However, *in situ* conservation does not always result in improved adaptation to pathogen virulence, suggesting a more complex evolutionary scenario, consistent with several published examples of plant–pathogen co-evolution in wild systems.

Introduction

In evolutionary biology, the Red Queen metaphor likens the process of evolutionary adaptation to a race in which the population (runner) must constantly move just to remain in the same place (Van Valen 1973). This notion has become central in conservation biology as well, and a number of evolutionary studies have stressed the importance of continued adaptation in preventing extinction (Gomulkiewicz and Holt 1995; Bell and Gonzalez 2009). A related consideration is the concept of 'lag load'. Under changing environmental conditions, the mean phenotype of a population is expected to track, but lag behind, the shifting phenotypic optimum because of the time required for the population to respond to selection (Lynch and Lande 1993). Lag load becomes a particular concern in the conservation of populations that are removed from their natural environment for a number of generations and may thus exhibit an even more pronounced lag in adaptation, because such isolation adds an additional period during which selection cannot track the phenotypic optimum (Schoen and Brown 2001).

A specific and common example of a situation where removal from co-evolutionary interactions is expected to reduce population fitness occurs within the area of crop genetic resource conservation. Seeds from agricultural species ('crop germplasm') are typically collected and stored in institutional seed banks, a mode of conservation referred to as '*ex situ*' conservation. *Ex situ* conservation provides reserves of seeds for breeders and/or for reintroducing varieties in the event of environmental or political catastrophes that lead to the loss of local crop germplasm (Clarke 2003; Elina et al. 2005; Fowler 2008). However, the isolation of crop germplasm from the environment and the resulting lag load could, in theory, result in significantly reduced fitness upon reintroduction. For this

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reason, *in situ* conservation, where crop germplasm is conserved on-farm, has been proposed as being an important complement to *ex situ* conservation because it is assumed to maintain the processes of co-evolution and adaptation of plant populations to changing biotic and abiotic conditions in a way that is not possible in a seed bank (Frankel 1974; United Nations 1992; Hamilton 1994; Secretariat of the Convention on Biological Diversity 2010).

In recent years, a number of studies have highlighted the importance of considering an evolutionary framework both in the study and in the conservation of crop germplasm (Thrall et al. 2010, 2011). Issues including adaptation of crop germplasm to climate change (Mercer and Perales 2010), the interaction between altitudinal gradients and local adaptation of maize (Mercer et al. 2008) and the potential for weeds and invasive species to evolve from domesticated plants (Ellstrand et al. 2010) have all been explored. Few studies, however, have explicitly tested the prediction that crop varieties conserved *in situ* are better adapted to current abiotic and biotic conditions compared to related *ex situ* collections. Exceptions include documented changes in the flowering date and drought stress tolerance of rice varieties in response to agricultural intensification (Tin et al. 2001), and changes in flowering date and morphological traits of Hopi maize varieties (Soleri and Smith 1995). We are unaware of any studies that have tested for changes in plant adaptation to fungal pathogens under *ex situ* and *in situ* conservation. Maintaining adaptation of crop germplasm to fungal pathogens and emerging pathogen strains is a particular concern because fungal pathogens of crops cause economically and socially important yield losses worldwide (Burdon and Thrall 2008).

There are two broad categories of host-plant resistance to fungal pathogens: qualitative resistance (also referred to as 'race-specific' or 'gene-for-gene') and quantitative resistance (also referred to as 'polygenic' or 'partial'). Qualitative resistance determines the ability of a specific pathotype of the pathogen to infect a specific genotype of the host plant. When resistance is effective, there is no infection when plants are exposed to the pathogen. However, if resistance fails, infection typically results in a disease phenotype. Pathogens and hosts co-evolve on a gene-for-gene basis (Flor 1956), with reciprocal selection of host resistance genes (R-genes) (Paillard et al. 2000a,b) and pathogen virulence genes (Wolfe et al. 1992). Novel virulence alleles that overcome host resistance increase pathogen fitness and may reach high frequencies in the pathogen population. There is, however, a fitness cost associated with virulence alleles that could select for their loss if a corresponding resistance disappears in the host population (Cruz et al. 2000; Burdon and Thrall 2003; Tian et al. 2003), and this limits the accumulation of virulence genes in the pathogen. The number of R-genes in the host plant is limited by the genetic architecture of the host (Wei et al. 1999) and allelism (Wei et al. 2002). *De novo* mutations or gene flow can also introduce novel alleles into the host or pathogen population, and recombination can result in novel combinations of virulence to host R-genes (i.e., novel pathotypes) as well as novel combinations of host resistance to the pathogen. This may result in an 'evolutionary arms race', and in this context, the maintenance of host-plant resistance is expected to be dependent on continued selection by, and adaptation to, the evolving pathogen population.

The second category of resistance, quantitative resistance, involves several genes that limit the spread of infection once gene-for-gene resistance has been overcome by the pathogen. Both types of resistance are important, but quantitative resistance is considered more durable as it is not pathotype-specific but is effective against all pathotypes of a pathogen species (Chelkowski et al. 2003). The genes for qualitative resistance sometimes map to the same quantitative trait loci as those for quantitative resistance (Maroof et al. 1994; Falak et al. 1999), suggesting that some of the same genes may be involved in both systems (Toojinda et al. 2000; Backes et al. 2003; Shtaya et al. 2006; Shen et al. 2007; Poland et al. 2011). However, the presence of high qualitative resistance does not necessarily imply high quantitative resistance to a specific isolate and *vice versa* (Wenzel et al. 2001).

There are two possible and contrasting outcomes regarding qualitative resistance of host-plant collections maintained *ex situ* versus *in situ*. First, *ex situ* collections (which, by virtue of storage as seed, are therefore not exposed to the current virulence combinations in the pathogen population) may lack the R-genes that match new pathogen virulences that evolved after the germplasm was removed from the environment, and so may show decreased resistance compared to *in situ* populations. Second, genetic drift in the *in situ* host population (perhaps due to a population bottleneck associated with colonization or demographic instability) may lead to loss of R-genes, and so collections conserved *ex situ* may maintain resistance alleles that no longer present in the field population (and to which the pathogen has lost the corresponding virulence). This could lead to the opposite result, namely increased resistance in *ex situ* populations compared to *in situ* populations.

Although quantitative resistance is not directly connected to R-genes, different pathotypes vary in their aggressiveness to the host. Therefore, in the case of quantitative resistance, recurring mass selection by farmers of the healthiest plants is expected to maintain the

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quantitative resistance of the *in situ* populations relative to *ex situ* populations.

Here, we study the relationship between conservation strategy and host-plant resistance to a pathogen. Our test system is a traditional variety of barley (*Hordeum vulgare* L. subsp. *vulgare*, hereafter referred to as *Hordeum vulgare*) together with the causative agent of powdery mildew of barley, *Blumeria graminis* (DC.) Golovinex Speer f. sp. *hordei* Marchal (hereafter referred to as *Bgh*), in Northern Morocco. We used detached-leaf (*in vitro*) assays and field trials to measure changes in the qualitative and quantitative *Bgh* resistance barley germplasm conserved both *in situ* and *ex situ* in geographically paired collections. We also characterized the R-genes present in both of the *ex situ* and *in situ* germplasm collections.

Materials and methods

Seed collections

Seeds of the traditional barley variety '*Beldi*', collected from five separate locations in Morocco in 1985, denoted Tao.1, Tao.3, Tao.4, Tao.5, and Tao.6 (Fig. 1) and conserved *ex situ* at ICARDA (International Center for Agricultural Research in the Dry Areas, Aleppo, Syria) were used as one set of materials for this study. The collection sites were located in Taounate Province and spanned a range of altitudes and climatic conditions. For the second set of materials, we re-sampled seeds of the '*Beldi*' variety from the same five locations in 2008, 23 years after the original collection date. To do this, we sampled seeds within a 3-km radius of each original sampling location. We collected at least five samples from each of four different fields within each site, to replicate the sampling strategy that was used to collect the initial samples in 1985 (Perrino et al. 1986).

Maternal effects and long storage may cause phenotypic differences between the progeny of seeds from seed bank collections compared to seeds collected in the field (Dreiseitl 2007). To remove these effects, we multiplied seeds from the original sample materials of both collections in the McGill University Phytotron in 2008–2009 ($N = 200$, i.e., 20 samples per site, per year). Seeds were disinfected with a 2.5% sodium hypochlorite solution and washed in sterile dH_2O to suppress possible fungal contamination. Seeds were planted in eight-inch pots and were randomly assigned to one of 20 blocks (4×5). Greenhouse conditions were 14:10 h L:D, 21:18°C and 1000 μmol/m^2/s photosynthetically active radiation at midday. Plants were fertilized weekly with 20:20:20 NPK solution beginning at anthesis [growth stage (GS) = 61] (Zadoks et al. 1974) and continuing through the end of grain filling (GS = 79). Seeds were then harvested from each individual plant, and equal numbers of seeds from each plant were used in subsequent tests.

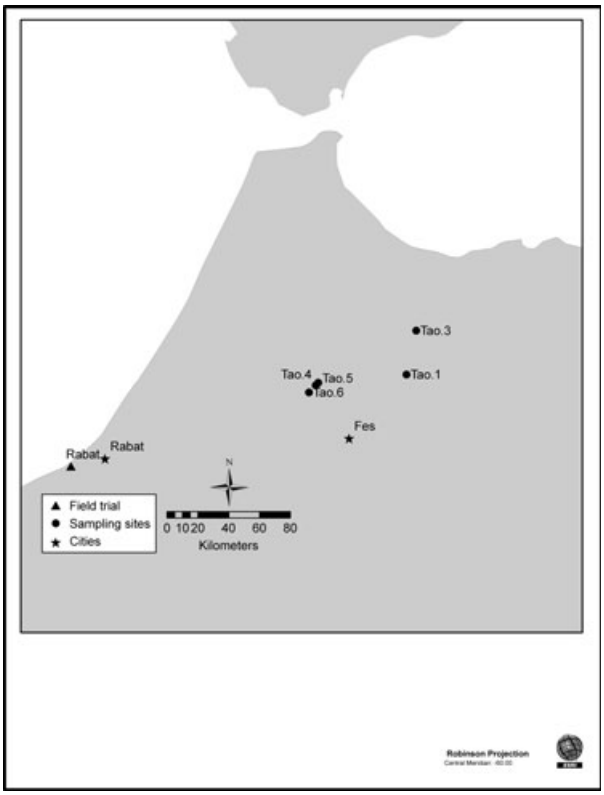


Figure 1 Sampling sites in Morocco for barley seeds and *Bgh* isolates as well as for conducting the field trial. Circle symbols (●) denote sites sampled for barley seeds in 1985 (by ICARDA) and re-sampled in 2008 (site codes are Tao.1, Tao.3, Tao.4, Tao.5, and Tao.6). The triangle symbol (▲) shows the site of the field trial in Rabat. Sampling sites for *Bgh* isolates correspond to the barley sampling sites Tao.1, Tao.3, Tao.4, Tao.6, as well as Rabat. Site agro-climatic zones are defined according to the system of the United Nations Food and Agriculture Organisation where the first two letters denote the moisture regime (SH, subhumid; SA, semi-arid), the second letter denotes the winter type (C, cool; M, mild), and the third letter denotes the summer type (W, warm) (FAO 2006). Site Tao.1 is zone SH-M-W, site Tao.3 is zone SH-C-W, sites Tao.4, Tao.5, and Tao.6 are SA-M-W, and Rabat is SH-W-W. The lowest altitude site (Rabat) is located at 23 m, and the highest altitude site (Tao.3) is located at 796 m.

Verification of seed collections using morphology and microsatellites

The hypothesis we test is whether plant populations maintained by Moroccan farmers under field conditions in specific locations continued to evolve resistance to the resident powdery mildew pathogen populations, compared with the same ancestral populations that had been earlier stored *ex situ* in the seed bank (i.e., away from the pathogen) for 23 years. We were concerned that, during these 23 years, different varieties of barley could have been intentionally introduced on some farms and replaced the original material. If that were the case, the

comparison(s) in question would not be examining the evolution of resistance in the *in situ* versus *ex situ* host-plant populations, but instead, the change in resistance brought about by the introduction of new germplasm. While the human-mediated flow of new germplasm from one site to another is frequently a component of *in situ* conservation occurring in traditional farming systems, our principle objective in this study was to compare the evolution of materials maintained *ex situ* with those same materials maintained under field conditions in specific locations. Hence, we felt it important to exclude from analysis any sample pairs in which there was evidence of human replacement of the *in situ* maintained material.

We therefore used both morphological and microsatellite data to compare each paired collection to identify evidence of replacement. We first compared six qualitative morphological traits used for describing barley varieties: row number, spike density, lemma awn, lemma awn barbs, glume/glume awn length, and rachilla hair length (Murphy and Witcombe 1986; IPGRI 1994). Seeds collected from 1985 and 2008 had identical distinguishing qualitative characteristics indicating that they belong to the '*Beldi*' variety. '*Beldi*' is a traditional, six-row variety, with a white aleurone layer, lax spike density, lemma awns, barbs along the entire length of lemma awns, glume plus glume awns of equal length to the kernels, and long rachilla hairs (Table S1). In the 2008 collections, we did find a small proportion of plants (<1%) belonging to two other traditional varieties: '*Roumi*' (a six-row variety distinguished by dense spikes and smooth awns) and '*Rouiza*' (a two-row variety). These samples were excluded from this study.

We then compared collections using three quantitative morphological traits: spike length, seed length, and number of triplets per spike. We compared the values of these traits between years using a one-way ANOVA. The means were not significantly different between years (Figure S1). These data provide an additional indication that there have not been any significant changes in the identity of the *Beldi* variety between the two collection years.

Finally, we used six microsatellite markers to test for genetic divergence between years. We collected leaf tissue from ten plants per sample, per year. Total genomic DNA was extracted from the leaf tissue using the DNA plant mini kit (QIAGEN, Toronto, Canada). Samples were characterized for the microsatellite markers Bmag0013, Bmag0321, Bmag0345, Bmac0316, Bmag0125, and EBmac0541 (Ramsay et al. 2000) conducting PCR with M13-tailed primers and a universal primer [M13 (-43)] (Schuelke 2000) labeled with IRDye700 or IRDye 800 (Li-Cor). Primer sequences and PCR conditions are provided as Appendix S1 (Tables S2 and S3). PCR products were separated using acrylamide gel electrophoresis on an Li-Cor sequencer. Allele sizes were determined using SAGA software (Li-Cor). Genetic divergence (Fst) was calculated between pairs of samples taken from the same site, in different years, using Arlequin 3.1 software (Excoffier et al. 2005).

The Fst values at several loci indicated that approximately 25% of the samples from 2008 differed significantly from those of the 1985 samples collected from the same sites. This observed divergence likely reflects high rates of human-mediated gene flow into the sites (e.g., arising from replacement of seed stocks by farmers). Thus, these samples were excluded from the remainder of the study because they likely were exposed to pathogen conditions that were not representative of those in the seed collection sites. The population pairs that we retained for this study had not significantly differentiated in genotype frequencies between years as measured by Fst (Table S4).

Overall, based on the combined comparisons of morphological and genotypic data, apart from the samples excluded, there was little evidence that new varieties had been introduced into the 2008 sites since the 1985 collection. This validates the use of these collections from different years to study changes in resistance over time in the resident populations.

Collection and pathotype determination of *Bgh* isolates

In the field, single lesions were removed from excised leaves and transported to the laboratory, where they were maintained on five percent agar containing 40 ppm of benzimidazole (Alfa Aesar, Lancaster, UK), a senescence inhibitor. Isolates were kept in a growth chamber at 12:12 h L:D, light intensity of 17 μmol/m^2/s, and temperature 18 ± 1°C. When sporulation occurred, 2.5-cm leaf sections of the susceptible barley variety Rabat 071 (Wiberg 1974) excised from seedlings (two-leaf stage) were inoculated by shaking spores from infected leaves onto the fresh leaves. When these lesions sporulated, single-spore isolates were placed on fresh leaves. This process was repeated twice to obtain monospore isolates. The culture was maintained by inoculating fresh leaves of Rabat 071 at every 8–9 days.

In February 2008, we collected *Bgh* isolates in the sites where barley samples had been collected. Because two of the barley collection sites (Tao.4 and Tao.5) are very close together, we considered them to represent only one collection site for *Bgh*, and therefore, we collected two isolates from each of four sites (Tao.1, Tao.3, Tao.4/Tao.5, and Tao.6), for a total of eight isolates of *Bgh* (Fig. 1). These *Bgh* isolates were collected for the purpose of testing the qualitative resistance of the traditional barley varieties to sympatric *Bgh* isolates using *in vitro* detached-leaf assays.

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The collection sites for these isolates were within 3 km of the collection sites for barley. We also sampled four isolates from the field plot in Rabat and an additional 15 isolates in the surrounding region to examine the diversity of *Bgh* pathotypes causing disease in field conditions. These isolates were used as indicators of the virulence gene combinations (pathotypes) present in the field trial for quantitative resistance and for comparisons with the isolates used in trials for qualitative resistance.

To determine the pathotypes of the isolates, we used detached-leaf assays with a differential set of 24 barley varieties, including 22 Pallas near-isogenic barley lines (NILs) (Kolster et al. 1986) as well as the barley varieties Lotta and Triumph (Table 1). The (NILs) share the same genetic background (Pallas) but vary for the presence of specific resistance genes (R-genes) or combinations of R-genes. If an isolate can successfully infect a Pallas NIL containing a specific R-gene, that isolate is considered to be virulent toward that specific R-gene, following the gene-for-gene concept. Conversely, failure to infect denotes avirulence to the R-gene. By compiling the virulence/avirulence of an isolate to the entire differential set, the pathotype is deduced.

Table 1. Differential varieties of barley and their associated resistance genes.

Differential	Resistance gene(s)*	Triplet†
Pallas	*Mla8*	1
P02	*Mla3*	1
P03	*Mla6*, *Mla14*	1
P04A	*Mla7*, *Mlk1*, *MlaNo3*	2
P04B	*Mla7*, *MlaNo3*	2
P06	*Mla7*, *MlaMu2*	2
P07	*Mla9*, *Mlk1*	3
P08A	*Mla9*, *Mlk1*	3
P08B	*Mla9*	3
P09	*Mla10*, *MlaDu2*	4
P10	*Mla12*, *MlaEm2*	4
P12	*Mla22*	4
P13	*Mla23*	5
P14	*Mlra*	5
P15	*Ml*(*Ru2*)	5
P17	*Mlk1*	6
P18	*Mlnn*	6
P20	*Mlat*	6
P21	*Mlg*, *Ml*(*CP*)	7
P22	*mlo5*	7
P23	*MlLa*	7
P24	*Mlh*	8
Lotta	*Ml*(*Ab*)	8
Triumph	*Mla7*, *Ml*(*Ab*), *MlaTr3*	8

*Gene names in parentheses [e.g., *Ml*(*Ru2*)] refer to R-genes have not yet been genetically mapped.
†Triplet assignment used for coding the pathotype.

We grew seedlings of the differential set in the greenhouse for 2 weeks until they reached the two-leaf stage. Individual plant pots were covered with cellophane bags during this time to prevent accidental inoculation by airborne *Bgh* spores (while still permitting air circulation). Primary leaf segments measuring approximately 2.5 cm were excised from seedlings and placed in Petri dishes of agar containing 40 ppm benzimidazole (a senescence inhibitor). A settling tower (10×25 cm) was used to inoculate Petri dishes with a single-spore *Bgh* isolate with an inoculation density of approximately 8 conidia/mm (verified visually). Petri dishes were then placed in the growth chamber at 12:12 h L:D and temperature 18 ± 1°C. Reaction types (RTs) were scored after 8–9 days using the nine-point 0–4 scale (including intermediate types) of (Torp et al. 1978) (Table S5). All assays were replicated twice and contained a susceptible (Rabat 071 variety) and resistant (Taffa variety) control to verify the efficacy of inoculation (we obtained these varieties from the Institut National de Recherche Agronomique (INRA), Rabat, Morocco). In accordance with standard practice, RTs 0 through 3 were classified as isolate avirulence (coded as '0'), while RTs 3–4 and four were classified as isolate virulence (coded as '1') (Dreiseitl and Wang 2007). These data were transformed to octal notation, where binary data for triplets of differentials is transformed to a single number to produce a numeric designation (the pathotype) representing each combination of virulence and avirulence genes (Limpert and Muller 1994).

To compare the virulence of the isolates found in the field site to that of isolates used for detached-leaf assays, we calculated frequency of virulence to each R-gene or R-gene combination as $p = x/N$, where x is the number of times the virulent RT was detected and N is the total number of samples tested. We calculated binomial variance of R-gene frequency and compared 95% confidence intervals of the virulence frequencies for the two sets of isolates.

Qualitative resistance of *ex situ* and *in situ* collections

Qualitative (gene-for-gene type) resistance of the barley collections to isolates of *Bgh* was assessed using detached-leaf assays, as described in the section on pathotype determination. In this case, however, we grew seedlings from both the 1985 (*ex situ*) and 2008 (*in situ*) collections. In the Petri dishes, we included control leaf segments of a susceptible variety (Rabat 071) and a resistant variety (Taffa) to verify the efficacy of inoculation and spore viability. The inoculated Petri dishes were then incubated and RTs were assessed, as previously described. An average of 10 barley seeds per site were tested against

each of the eight *Bgh* isolates [two isolates from each of the *Bgh* sampling sites (Tao.1, Tao.3, Tao.4/Tao.5, and Tao.6)] for a total of 768 inoculations. All assays were replicated twice to verify the RT. In the case of a discrepancy between replicates, a third test was conducted and the more frequent RT was used. RTs from 0 to 2–3 were categorized as resistant (R), while RTs from categories 3 to 4 were categorized as susceptible (S). The observed frequency of R and S RTs was calculated for each combination of barley collection, *Bgh* isolate, and year. We tested for differences in the distribution of RTs between years for each collection and isolate using a chi-squared test.

Qualitative resistance: R-genes present in barley

To determine the R-genes present in the barley samples, we used a set of 50 reference isolates of *Bgh* held in the pathogen genebank at Agricultural Research Institute Kromeriz (Czech Republic). Octal pathotype designation of the isolates was derived from their virulence patterns corresponding to twelve *Ml* resistance genes in coded triplets (Limpert and Muller 1994) in the following order: *a1*, *a3*, *a6*; *a7*, *a9*, *a12*, *a13*, *k1*, *La*, *g*, *at*, and (*Bw*) (Table S6).

Detached-leaf assays were conducted at Agricultural Research Institute Kromeriz. We tested 42 samples from 1985 and 42 samples from 2008, in the sites Tao.1, Tao.3, Tao.4, Tao.5, and Tao.6. Approximately 50 seeds of each barley accession were sown in two pots (80 mm diameter) filled with a gardening peat substrate and placed in a mildew-proof greenhouse under natural daylight. Leaf segments 20 mm long were cut from the central part of healthy fully expanded primary leaves. Three leaf segments of each accession were placed in a Petri dish on water agar (0.8% and 40 ppm benzimidazole) for testing with each isolate. Before inoculation, each isolate was purified, verified for the correct virulence phenotype on standard barley lines, and increased on leaf segments of a susceptible line B-3213. For each isolate, a Petri dish with leaf segments was placed at the bottom of a metal inoculation tower and inoculated at an inoculum density of ca. 8 conidia/mm. The dishes with inoculated leaf segments were incubated at 18 ± 2°C under artificial light (cool-white fluorescent lamps providing 12-h light at 30 ± 5 μmol/m^2/s).

Eight days after inoculation, RTs on the upper part of the adaxial side of leaf segments were scored on a nine-point scale, as previously described. Each cultivar was tested in two replications. In the rare cases where leaf segments with more than one distinct RT occurred, the more frequent RT was used for analysis. A set of 50 RTs for each isolate provided the basis for a resistance spectrum (RS) (i.e., a compilation of the R-genes present) of each cultivar. RTs from 0 to 2–3 were categorized as plant resistance (R), indicating the presence of the given R-gene, while RTs from categories 3 to 4 were categorized as susceptible (S), indicating the absence of that R-gene. The resistance in each cultivar was postulated by comparing the RS with previously determined resistance spectra of standard barley lines possessing known resistance genes.

The presence or absence of each R-gene was treated as a binomial variable. Because the samples are all homozygous (owing to high levels of natural inbreeding in this species), this can be considered equivalent to a haploid model. We calculated the frequency and binomial variance of each R-gene per site and per year. We tested for differences in R-gene frequency between years (with sites as replicates) using Student's t-test. To compare the distribution of the different resistance spectra in the *in situ* and *ex situ* collections, we calculated the frequency of each RS in each collection year and compared them using Student's *t*-test.

Quantitative resistance: field trial

Quantitative (polygenic) resistance of the seed collections to *Bgh* was assessed in a field trial that measured powdery mildew infection and disease progression on barley plants. The field trial was conducted at Institut Agronomique et Vétérinaire Hassan II (Rabat, Morocco) from January to March 2009. We tested geographically paired samples from the collection sites. There was one pair of samples from each of sites Tao.1, Tao.3, Tao.4, and Tao.6 and two pairs of samples from site Tao.5, for a total of 12 samples (six samples from 1985 and six samples from 2008). A randomized complete block design, with a total of five blocks, was used. Each block contained one replicate of each of the twelve samples. Samples were planted in 15-seed hill plots (Walsh et al. 1976) with a distance of 30 cm between hills for each experimental unit. Infection with *Bgh* occurred via natural inoculation. All blocks were separated by three rows of a susceptible spreader variety (Rabat 071), and the entire plot was surrounded by three rows of Rabat 071 to increase natural inoculation and spreading of infection.

The percentage of powdery mildew infection on the first four leaves of plants was estimated visually. Infection was assessed weekly for five plants per hill plot once plants reached the four-leaf stage (GS = 14) (James 1971). A total of five readings were taken. Prior to estimating the percentage of *Bgh* infection in the field, training of field workers for the accurate visual estimation of the leaf area covered with powdery mildew was conducted using DISTRAIN, a software program that evaluates the accuracy and consistency of visual estimation of disease

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coverage (Tomerlin and Howell 1988). Training was completed when all readings were at least 90% accurate. In the field, one person conducted all the readings to further minimize variation. Readings were 'blind', meaning that the person did not know the identity of the sample being scored, to eliminate potential bias. Area under the disease progress curve (AUDPC) was calculated as follows:

$$\sum_{i=1}^{n} [(Y_{i+n1} + Y_i)/2][X_{i+1} - X_i]$$

where Y_i is mildew severity (per unit) at the ith observation, X_i is the time in days at the ith observation, n is the total number of observations and n_1 is the first observation (Shaner and Finney 1997; Jeger and Viljanen-Rollinson 2001). AUDPC values were analyzed using a factorial ANOVA with collection year and collection site as factors and with a randomized complete block design. Pairwise comparisons were made using Tukey's HSD test.

Results

Characterization of *Bgh* isolates used in the qualitative and quantitative resistance trials

The *Bgh* isolates used for the qualitative resistance assay (*in vitro* assays) had a high virulence complexity, with 9–11 virulence genes per isolate. There were seven distinct pathotypes (combinations of virulence genes) among the eight isolates, with pathotype 60042706 occurring in both sites Tao.1 and Tao.6 (Table 2). Most isolates were virulent to the R-genes *a8*, *a3*, *a10* + (*Du2*), *ra*, *k1*, *nn*, *at*, *h*, and (*Ab*) (Figure S2).

The four isolates from the field plot all had distinct pathotypes with virulence to four to ten of the NILs per isolate (Table 2), indicating that field infection is caused by the simultaneous development of a genetically diverse population of *Bgh* spores rather than by a single pathotype that rapidly multiplied and infected the field. This is further supported by the pathotypes of the 15 *Bgh* isolates from the Rabat region near the field plot, all of which were unique and had virulence to resistance genes in six to eleven of the NILs per isolate. These isolates had similar pathotypes and virulence frequencies to those used in the detached-leaf assay (Figure S2). Overall, these isolates can be considered to have similar pathotypes, and the differences observed between the isolates should therefore not be a confounding variable when comparing the results of the field trials with the results of the qualitative resistance (detached-leaf) trials. The isolates characterized had different pathotypes and frequencies of R-genes than isolates described for the region in 1992 (Yahyaoui et al. 1997). This indicates that there has been evolution of isolate pathotypes in the region over the time period discussed in this study.

Table 2. Characteristics of the *Bgh* isolates used in the *in vitro* assays and in the field trial.

Isolate	Site	Pathotype*	Complexity†	Experiment‡
Pm.200	Tao.1	60013704	9	*In vitro* assays
Pm.202	Tao.1	60042706	9	*In vitro* assays
Pm.209	Tao.3	40012746	9	*In vitro* assays
Pm.034	Tao.3	70044614	9	*In vitro* assays
Pm.223	Tao.4/Tao.5	60042716	10	*In vitro* assays
Pm.234	Tao.4/Tao.5	60043706	10	*In vitro* assays
Pm.241	Tao.6	60042706	9	*In vitro* assays
Pm.068	Tao.6	60044706	9	*In vitro* assays
Pm.114	Rabat	20043704	8	Field trial region
Pm.116	Rabat	40040706	7	Field trial region
Pm.092	Rabat	40042004	4	Field trial site
Pm.113	Rabat	40042516	8	Field trial region
Pm.118	Rabat	40042704	7	Field trial region
Pm.090	Rabat	40042744	8	Field trial site
Pm.089	Rabat	40043716	10	Field trial site
Pm.106	Rabat	40046506	8	Field trial region
Pm.117	Rabat	40047506	9	Field trial region
Pm.115	Rabat	60010106	6	Field trial region
Pm.124	Rabat	60010704	7	Field trial region
Pm.123	Rabat	60012544	8	Field trial region
Pm.088	Rabat	60042504	7	Field trial site
Pm.120	Rabat	60042506	8	Field trial region
Pm.119	Rabat	60042507	9	Field trial region
Pm.107	Rabat	60042704	8	Field trial region
Pm.111	Rabat	60042706	9	Field trial region
Pm.112	Rabat	60052704	9	Field trial region
Pm.121	Rabat	70013546	11	Field trial region

*Each pathotype represents a distinct combination of virulence genes.
†The total number of virulent reaction types per isolate
‡'Field trial site' indicates that the isolate was collected from the actual field trial. 'Field trial region' indicates that the isolate was collected from the same region as the field trial.

Qualitative resistance of barley to *Bgh* isolates

The qualitative RTs (resistant and susceptible) to eight different *Bgh* isolates were compared for five separate geographically paired samples of *ex situ* and *in situ* conserved samples (Fig. 2). Of the 40 combinations of isolate and seed collection, there were five collection-by-isolate combinations that had a significant difference in resistance between *in situ* and *ex situ* collections. These were isolate Pm.34 and site Tao.3 (*ex situ* more resistant than *in situ*), isolate Pm. 68 and site Tao.3 (*ex situ* more resistant than *in situ*), isolate Pm.200 and site Tao.4 (*ex situ* more resistant than *in situ*), isolate Pm.223 and site Tao.5 (*ex situ* more resistant than *in situ*), and isolate Pm.234 and site Tao.1 (*in situ* more resistant than *ex situ*). In 13 of the 40 isolate-by-seed-collection combinations, all the seedlings from at least one of the collections were susceptible. Of these, in six

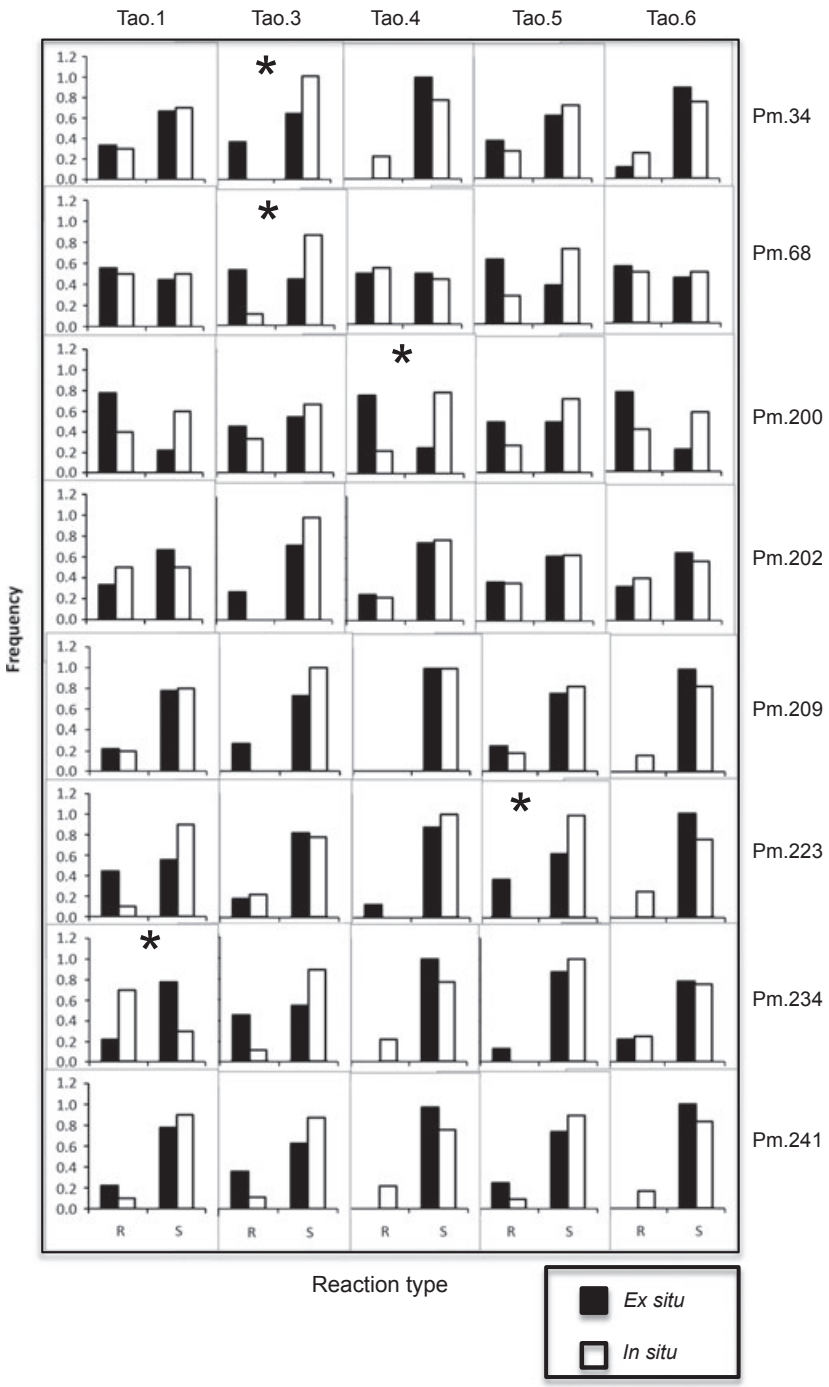


Figure 2 Qualitative resistance of geographically paired barley collections from 1985 (*ex situ*) and 2008 (*in situ*) to eight different *Bgh* isolates. Black bars denote *ex situ* collections, while white bars denote *in situ* collections. Asterisk indicate significant difference ($P < 0.05$) between the *ex situ* and *in situ* collections. There are two possible reaction types: resistant (R) or susceptible (S).

cases the *ex situ* collection was entirely susceptible, in another six cases, the *in situ* collection was entirely susceptible, and in one case, both *ex situ* and *in situ* collections were entirely susceptible.

Qualitative resistance: R-genes present in barley

The barley collections from 1985 and 2008 contained a total of 29 R-genes. Of these, 16 were present in 1985

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and 21 were present in 2008. Each individual accession tested had between 0 and 3 R-genes. The mean number of R-genes in the resistance spectra of the accessions remained similar between years, averaging 1.4 (±0.6) in 1985 and 1.6 (±0.6) in 2008. The frequencies of the resistance genes that were present in both years (and could therefore be compared using a *t*-test; resistance genes *a8*, *at*, (*Ch*), *u3*, *u11*, *ra*, and *h*) did not change significantly between years. Resistance gene *a8* was the most common (frequency = 0.6 in both 1985 and 2008) and was present in both years and in all sites (Fig. 3). The resistance gene '*g*' was detected for the first time in 2008, where it occurred at intermediate frequencies (0.22 and 0.33) in sites Tao.1 and Tao.6, respectively. A total of 16 distinct, but unidentified, R-genes (*u1–u16*) were present in the germplasm at low frequencies (0.10 or less).

The *a8* gene occurred in the most common resistance spectra (i.e., combinations of resistance genes in a single sample), either alone or in combination with other R-genes, including *at*, *u3*, and *u11*, (Fig. 4). There were eight resistance spectra that were present in both years: *a8*; (*Ch*); *a8* + *u11*; *a8* + *at*; *a8* + *u3*; *u16*; *at*; and 'none', the latter indicating accessions with no detected R-genes. The frequencies of these resistance spectra were not significantly different between years in *t*-tests. In contrast to the common R-genes and their resistance spectra, the identity of rare resistance genes and rare resistance spectra showed a complete turnover between 1985 and 2008. This is not unexpected by chance alone, given the low frequencies of these genes.

Quantitative resistance of barley to *Bgh*

AUDPC was used as a measure of quantitative disease resistance in field trials. AUDPC values ranged from 37.6 to 719.0, with a median value of 287.1 (higher AUDPC indicates lower quantitative resistance). ANOVA revealed that both collection year ($F = 18.3923$, df = 1, $P = 0.0001$) and sampling site ($F = 6.2046$, df = 5, $P = 0.0002$) were significant factors with regard to AUDPC, as was the interaction term ($F = 23.7394$, df = 5, $P < 0.00001$) (Table 3). In two of the 2008 (*in situ*) collections (sites Tao.3 and Tao.6), quantitative resistance was significantly greater than in the respective paired 1985 (*ex situ*) samples, whereas for one of the collection pairs (site Tao.5.1), the *ex situ* collection had higher resistance (Fig. 5).

Discussion

We identified differences in *Bgh* resistance between barley collections conserved *in situ* and *ex situ.* These differences only rarely involved the gene-for-gene system (qualitative resistance) of host–pathogen interactions (Jones and Dangl 2006) with only 5 of 40 significant differences in resistance between collections. This was reflected in the lack of differences in the frequencies of major R-genes in the host germplasm, although minor genes changed in identity and the *Mlg* R-gene appeared in the *in situ* germplasm. We also found changes in quantitative resistance between some of the collections in the field trials, with

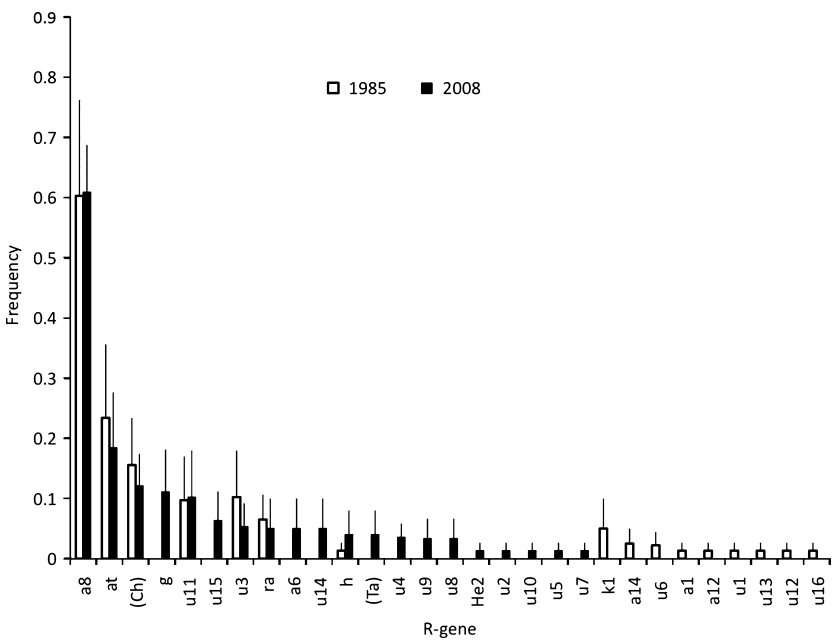


Figure 3 Resistance genes present in barley samples from 1985 (*ex situ*) and 2008 (*in situ*). Bars represent the mean frequency of the five sites (+SE). There were no significant differences in frequencies between years.

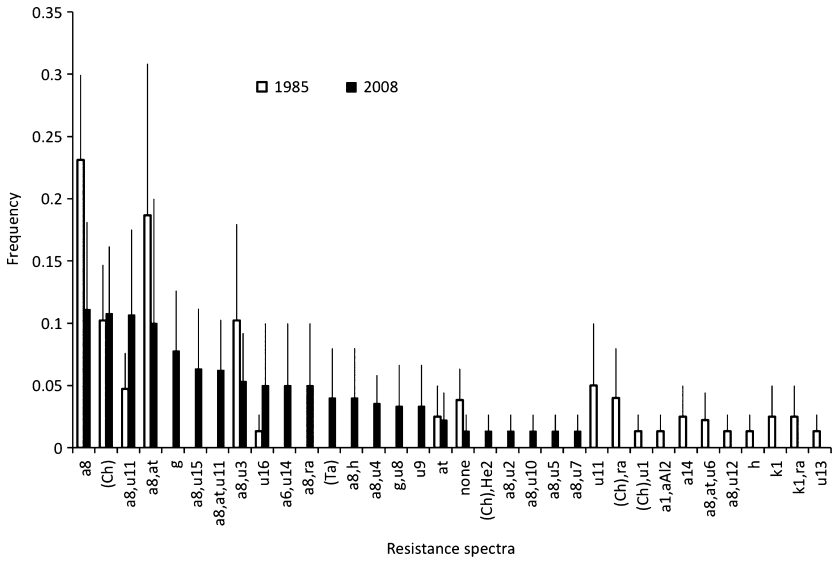


Figure 4 Resistance gene spectra (combinations of resistance genes) present in barley samples from 1985 and 2008. Bars represent the mean frequency of the five sites (+SE). There were no significant differences in frequencies between years. Some samples contain only one R-gene, resulting in a resistance spectrum with only one R-gene. R-genes with prefix 'u' represent genes that are unidentified and are known only by the reaction spectrum of reference barley varieties to reference *Bgh* isolates. R-gene names in parentheses [e.g., *Ml*(*Ru2*)] refer to R-genes that have been identified in specific genetic backgrounds but have not yet been genetically mapped.

Table 3. Two-way analysis of variance for area under the disease progress curve testing the effects of year collected (i.e., 1985 vs 2008) and collection site.

	df	Sum square	Mean square	*F*-value	*P*
Year	1	133058	133058	18.3923	<0.0001
Site	5	224433	44887	6.2046	<0.0001
Year × site	5	858703	171741	23.7394	<0.0001
Residuals	44	318314	7234		

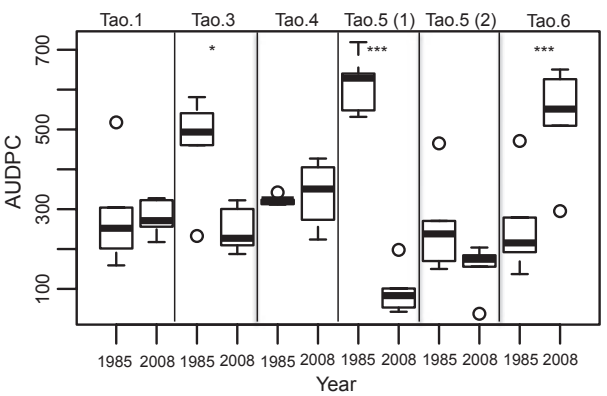


Figure 5 Quantitative resistance of paired barley collections from 1985 (*ex situ*) and 2008 (*in situ*) to *Bgh*. Resistance is measured as area under the disease progress curve following natural inoculation with *Bgh* in field plots in the Rabat region. Each panel denotes collections from one site compared between years. Asterisks denote significant differences between *ex situ* and *in situ* samples from a given site (*$P < 0.05$, ***$P < 0.001$).

the highest quantitative resistance being in the *in situ* collections.

These findings provide some support for our original expectations, namely that qualitative resistance can either decrease *ex situ* (owing to lag load exacerbated by storage of germplasm away from the natural habitat) or increase *ex situ* (owing to rare R-genes being preserved *ex situ* but lost *in situ*), while quantitative resistance should increase in the *in situ* collections. We saw evidence of both of these expected trends in both types of resistance. It is of note that *ex situ* collections appeared to conserve a greater number of adaptive genes for qualitative resistance compared to *in situ* collections, possibly due to the preservation of rare R-genes to which the pathogen has lost virulence. This is contrary to the general assumption that only *in situ* collections maintain adaptive resistance and confirms our hypothesis that removal from the environment may, in some cases, provide a mechanism to conserve useful and adaptive resistance. In other cases, *in situ* conservation resulted in higher resistance, consistent with the established paradigm. However, the small number of differences between *in situ* and *ex situ* germplasm for qualitative resistance was unexpected. With the possibility of type I error expected to account for approximately two of these significant results, the number of significant differences is effectively very small.

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One possible explanation for the small number of differences in qualitative resistance between *in situ* and *ex situ* collections is that the evolutionary dynamics of the host–pathogen interaction studied here do not conform as closely as expected to the classic gene-for-gene model. In order for gene-for-gene interactions to bring about an 'evolutionary arms race', there must be sufficient selective pressure and sufficient genetic variation in the co-evolving system (Bergelson et al. 2001). Selective pressure is provided by the presence of disease (causing yield losses of up to 30% in North Africa) (Caddel and Wilcoxson 1975; Amri et al. 2002) and the frequent appearance of novel virulence within the pathogen population. Resistance variation is provided by a large pool of R-genes in the host populations or by the capacity to acquire these new genes via gene flow or *de novo* mutation. The pathogen in our study conformed to these assumptions and exhibited significant turnover in pathotypes within the time period studied (Yahyaoui et al. 1997; Jensen et al., unpublished data). However, the host apparently did not conform well to these expectations. The host population had a high number of resistance genes but lacked several major genes identified in other populations of traditional Moroccan barley such as *a12* and *a6* + *a14* (Czembor and Czembor 2000). Furthermore, most of these genes were present at low frequencies. This relatively low diversity and frequency of R-genes within the host population may have reduced the strength of frequency-dependent selection and cycling of R-genes and virulence genes over the 23-year period of this study.

Another possible reason why we did not frequently detect lower resistance in the *ex situ* maintained materials could be our restriction of comparisons to collections pairs where we were confident that the *in situ* collection had evolved in the same site as the *ex situ* collection (based on Fst values). As discussed earlier, this was done to avoid comparing *ex situ* maintained materials with newly introduced on-farm germplasm that may have replaced those made in the 1985 collection and was not subject to selection pressures *in situ*. It is possible that divergence in microsatellite allele frequencies could have come about as the result of hitchhiking effects associated with selection for pathogen resistance that occurred in the 23 years separating the two collections. We believe this to be unlikely, for two reasons. First, linkage is weak between known R-genes and the microsatellites used in this study (Varshney et al. 2007; Aghnoum et al. 2010) (Table S2). Second, to drive Fst to the significantly different levels seen in the excluded materials (Fst = 0.2–0.3), hitchhiking and selection during the short intervening period when the two collections were made would have had to be intense for many R-genes.

The most abundant R-genes present in *ex situ* and *in situ* collections (*a8*, *at*, (*Ch*), *u*, *u3*) remained unchanged in frequency in both collections. The pathogens had high frequencies of virulence to R-genes *a8* and *at* [data for (*Ch*), *u*, and *u3* are not available], making these R-genes ineffective for preventing infection. However, there were several potentially adaptive changes in host resistance. The R-gene '*g*' increased in frequency from 0% in 1985 to 10% in 2008. Because 82% of the pathotypes in this region are avirulent to '*g*' (Jensen et al., unpublished data), this would be a relatively effective source of resistance and is a potentially adaptive change in the host germplasm. The R-gene *a6* was only identified in the *in situ* collections, and only one of the isolates was virulent to it. Despite these potentially adaptive changes, there does not appear to be a tight co-evolution between host R-genes and pathogen avirulence genes in the *in situ* populations.

Although the major R-genes remain unchanged, the rare R-genes in the germplasm, (mostly the unidentified '*u*' genes) showed complete turnover in identity, with 13 new R-genes detected in 2008 compared to 1985. There are two possible explanations for this result. First, gene flow from other collections, possibly European barley varieties, introduced new R-genes into the germplasm, while rare R-genes that are no longer present *in situ* were retained in the *ex situ* collection. Second, because these genes are rare, our sample was not large enough to detect them in both years. Further tests are required to determine which is the correct explanation.

With regard to quantitative resistance, our study identified significant differences between several of the geographically paired *in situ* and *ex situ* collections that we tested. Two of the *in situ* conserved collections showed higher quantitative resistance to *Bgh* than the associated *ex situ* collections. Conversely, one *ex situ* collection had higher quantitative resistance than the *in situ* collection. One of the *in situ* collections (from the Tao.6 site) had much lower disease levels than any of the other *in situ* or *ex situ* collections, indicating a potentially interesting and novel source of quantitative resistance derived via *in situ* conservation. These results are paralleled by reports in the breeding literature that bulked hybrid populations of crop plants planted year after year in the same site show an increase in quantitative resistance. This pattern is seen for barley and powdery mildew (Ibrahim and Barrett 1991), wheat and powdery mildew (Le Boulc'h et al. 1994; Paillard et al. 2000a,b) as well as for host–pathogen systems in other crops (Horneburg and Becker 2008). Although we have observed changes in quantitative resistance in two *in situ* collections, it is difficult to ascertain whether this is attributable to selection or is a by-product of neutral processes such as genetic drift or gene flow. However, in a wild host–pathogen system (flax and flax rust), quantitative resistance was subject to adaptive co-evolution in

a similar manner to qualitative resistance (Antonovics et al. 2011), despite the difference in genetic mechanisms. The evolutionary maintenance of quantitative resistance is an agronomically important trait for crop genetic resource management because it has the potential to confer durable resistance. Quantitative resistance indicates the action of genes that work after a successful infection by the pathogen to limit damage to the plant and induce resistance to further infection. This type of resistance is considered to be equally effective against numerous pathogen isolates and is less vulnerable to the evolution of novel virulence genes in the pathogen. For these reasons, plant breeders place a high value on quantitative resistance (compared to qualitative resistance) (Murphy et al. 2005).

It is of note that the majority of the barley samples tested were moderately or highly susceptible to *Bgh*. Furthermore, although 29 R-genes were detected, only four of these had frequencies above 10% and only one (*a8*) had a frequency above 50%. Farmers report that they use the *Beldi* variety because it is adapted to local conditions, including poor soil, rain-fed cultivation, and periodic droughts. Experimental data have demonstrated that traditional Moroccan varieties consistently outperform registered Moroccan varieties in terms of drought stress resistance (Oukarroum et al. 2007). The *Beldi* variety is also robust to the practice of green stage grazing (Anderson 1985; Yau and Mekni 1987) and yields high-quality straw used for livestock feed that represents a significant proportion of the crop value (Annicchiarico and Pecetti 2003; Grando et al. 2005). The finding that *Beldi* is susceptible to many *Bgh* isolates suggests that farmers could benefit from participatory breeding efforts to improve the qualitative and quantitative resistance of this germplasm while preserving these other desirable and culturally adapted traits (Bellon 1996; Brush and Meng 1998; Phillips and Wolfe 2005).

Overall, this study highlights different adaptive outcomes of conserving seeds *ex situ* compared to conserving seeds *in situ*. From the genetic resource management perspective, this demonstrates the importance and complementarity of the two types of conservation. Conservation policies should therefore include both *in situ* and *ex situ* conservation strategies. Furthermore, the differences we have seen between qualitative and quantitative resistance of collections underscore the importance of assessing both types of resistance when evaluating the efficacy of different conservation methods for maintaining pathogen resistance in crop germplasm. The maintenance of multilocus (quantitative) resistance to pathogen populations is likely best achieved by recurrent mass selection of a large and heterogeneous population and is thus more likely to occur on-farm (*in situ*). This process can potentially be enhanced using participatory and on-farm breeding techniques (Dawson et al. 2008). However, in some cases, *ex situ* collections may preserve some specific alleles that have been lost from *in situ* populations.

Future work on this topic should include expanding the components of this study to plant–pathogen systems in different traditional farming systems to test the generality of the conclusions regarding *in situ* and *ex situ* conservation. In particular, it would be interesting to examine crops with different mating systems, such as outcrossing and clonally propagated crops. Further study of changes in quantitative resistance of *in situ* and *ex situ* materials, with a larger number of samples and covering a broader geographic region, could provide important insights.

The emergence and dissemination of novel pathogen virulence and host resistance is a stochastic and unpredictable process that can have devastating impacts on crop yields. This was recently exemplified by the emergence of a novel race of wheat rust (UG99) in Uganda (Singh et al. 2007) with virulence the majority of the resistance genes deployed in cultivated wheat (Jin et al. 2009). This race has spread to the Middle East and has the potential to affect the major wheat-growing areas in Asia and potentially in North America (Nazari et al. 2009). In the case of this pathogen, the search for sources of resistance has encompassed both modern and traditional crop varieties (Sidiqi et al. 2009) and both qualitative and quantitative sources of resistance (Singh et al. 2011). Our study demonstrates, from an evolutionary perspective, the importance of maintaining both *in situ* and *ex situ* collections of traditional crop varieties for use in the defense against novel pathogen races. *In situ* collections had higher resistance in some cases and showed some potential changes in R-gene frequency and identity. Conversely, although some of the *ex situ* collections suffered from lag load, as could be expected under the Red Queen scenario, others had high resistance, and furthermore, they may retain rare resistance alleles that can be useful in future breeding applications.

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Data archiving statement

Data for this study are available as Supporting Information (Appendices S2–S4).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mean values of three morphological traits: number of triplets per spike (A), spike length (B) and seed length (C), for barley samples collected from the same five sites in both 1985 and 2008.

Figure S2. Virulence frequencies (+SE) of *Bgh* isolates from the Rabat region and the Taounate region (in the seed collection sites) to the resistance genes and resistance gene combinations in the differential barley varieties.

Table S1. Comparison of barley collections from 1985 and 2008 using qualitative and quantitative morphological traits.

Table S2. Names, chromosome, linkage to R-genes, primers sequences, PCR programs and product size range for the microsatellite markers used to characterize the *H. vulgare* samples.

Table S3. Details of the PCR programs used for the microsatellite markers.

Table S4. Comparison of the seed samples collected in 2008 to the original seed samples from 1985, using microsatellite data and AMOVA analysis. Note that all comparisons show non-significant Fst and therefore minimal genetic differentiation between sample pairs.

Table S5. Infection type classification for powdery mildew on cereals (from Torp et al. 1978).

Table S6. Octal pathotypes of the isolates used to characterize the resistance spectra of the barley accessions.

Appendix S1. Supplementary tables and figures providing information in support of the data presented in this study.

Appendix S2. Qualitative resistance data for barley conserved *in situ* and *ex situ*.

Appendix S3. Data for AUDP from the field trial.

Appendix S4. Data for R-genes present in traditional barley varieties.