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Genotypic variation in cold tolerance of 18 Ethiopian rice cultivars in relation to their reproductive morphology

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ABSTRACT

Male sterility induced by low temperatures during reproductive development is the major constraint on rice production in Ethiopia, which generally lies at high elevations. Because of a lack of phenotyping facilities, limited information is available on the cold tolerance of Ethiopian germplasm. We evaluated the genotypic variation in cold tolerance of 18 Ethiopian rice cultivars in two phenotyping facilities and characterized their cold tolerance in relation to their reproductive morphology in a 2-year trial in Japan. Genotypic variation in spikelet fertility was high after exposure to cold during reproductive development at both facilities, with fertility ranging from 0% to 90 %. 'Andassa' and 'Tana' had the highest fertility and 'Fogera 2' and 'Getachew' had the lowest. The two cold-tolerant germplasms had tolerance similar to that of the Japanese 'Hitomebore' (strong), whereas the susceptible germplasms had tolerance similar to that of the Japanese 'Sasanishiki' (weak). The variation in spikelet fertility was explained by both anther length and number of fertile pollen grains per anther under cold stress, and by anther length under unstressed control conditions in both years of the study; longer anther length and higher fertile pollen number leads to stronger cold tolerance. Our results suggest that anther length under unstressed conditions offers a pre-screening criterion for cold tolerance without requiring phenotyping facilities for screening.

1. Introduction

Ethiopia relies on imports to meet its growing demand for rice, with demand now exceeding 300 kt annually (FAO, 2020). Domestic production covers only about 30 % of this demand, with rice produced under a cool climate in the country's highlands, at elevations above 1800 m a.s.l., in the Fogera area of Ethiopia's Amhara region, a major rice-producing area that accounts for more than 40 % of the national production (Ministry of Agriculture and Rural Development, Ethiopia, 2010) (Fig. 1). In this area, cold damage is a serious constraint on rice production (Abera et al., 2020; Dessie, 2020). Minimum monthly temperatures during the rice cultivation season (June to November) in Ethiopia average less than 15 °C (Abera et al., 2020) and the daily minimum temperature sometimes drops below 12 °C. Since rice, which originated in the tropics, is sensitive to low temperatures during reproductive development (Shimono et al., 2002, 2007), even a slight temperature decrease below a threshold of about 17 °C, especially at the young microspore stage, inhibits pollen development and results in male sterility and severe yield losses caused by a shortage of pollen during the pollination phase of flowering (Satake, 1976; Shimono et al., 2002).

Genotypic improvement of cold tolerance during reproductive development is an important way to alleviate the yield loss caused by

Abbreviations: DAT, days after transplanting; FT, spikelet fertility; T_a, air temperature; T_w, water temperature.

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low temperatures. The degree of cold tolerance is under genetic control and varies widely among accessions (Farrell et al., 2006; Satake and Shibata, 1992; Shimono et al., 2016; Wainaina et al., 2015, 2018). In Ethiopia, 35 cultivars have been released and registered by screening more than 3000 accessions, which comprised inbred lines supplied mainly by the African Rice Center and the International Rice Research Institute (IRRI) until 2013 (Dessie, 2020). The screening was conducted in Ethiopia during field trials at specific locations that covered a range of environmental conditions, in multiple years, during cultivation seasons. This represents a reasonable and straightforward strategy to select good candidates that are adaptable to each environment, and can successfully identify cultivars with high cold tolerance. However, in addition to being expensive, it relies on unpredictable natural variation in weather within the range of the tested conditions and focuses on final grain yield; thus, some cultivars may attain high yield by escaping cold stress during the cultivation period rather than by resisting that stress (Abera et al., 2020). It's necessary to evaluate their cold tolerance under controlled conditions.

In evaluation of cold tolerance it is mandatory to precisely control the temperatures of the developing anthers and pollen, which are the organs most sensitive to cold and therefore responsible for male sterility (Satake, 1976). Cool irrigation during reproductive development in the field using the deep-water method (flooding to a depth of 30 cm) is widely used for phenotyping the cold tolerance of rice accessions (Farrell et al., 2006; Suh et al., 2010; Wainaina et al., 2015, 2018; Zhu et al., 2015). This method can evaluate a large number of genotypes, including segregated inbred lines, in rice paddies during breeding research. In fact, it has contributed to the continuous release of new cultivars with higher cold tolerance in Japan (Matsunaga, 2005) and has alleviated cold damage for farmers in northern Japan who adopted these cultivars (Shimono et al., 2010). For more precise physiological analysis, a method of evaluating the cold tolerance of the main stem after tiller removal has been used with pot-grown plants exposed to low water temperatures with the deep-water technique (Koumoto et al., 2016; Shimono et al., 2011). The pot conditions allow flexible manipulation of the duration of exposure and strength of the stress, while also accounting for differences among the accessions in the time to reach maturity. Unfortunately, facilities suitable for testing cold tolerance are rarely available around the world; most are located in Asian and Pacific countries such as Japan (Koumoto et al., 2016; Matsunaga, 2005; Shimono et al., 2011; Wainaina et al., 2015, 2018), China (Zhu et al., 2015), Korea (Suh et al., 2010), and Australia (Farrell et al., 2006). There are no such facilities in Africa. As a result, no information is available on the cold tolerance of Ethiopian germplasm.

Cold tolerance results from different physiological mechanisms that occur during the fertilization process and that differ among accessions (Shimono et al., 2016; Satake and Shibata, 1992). Accessions with higher tolerance to cold during reproductive development can maintain a higher number of engorged pollen grains per anther and longer anthers, thereby letting them attain sufficient pollination during the flowering stage. This results from one or more of the following three factors: (1) initiation of a greater number of pollen grains in the anthers during early reproductive development, which represents the potential number of pollen grains; (2) a higher survival rate of the pollen after exposure to cold, owing to high tolerance to stress by individual pollen grains; and (3) higher pollination success (i.e., ability of the pollen to

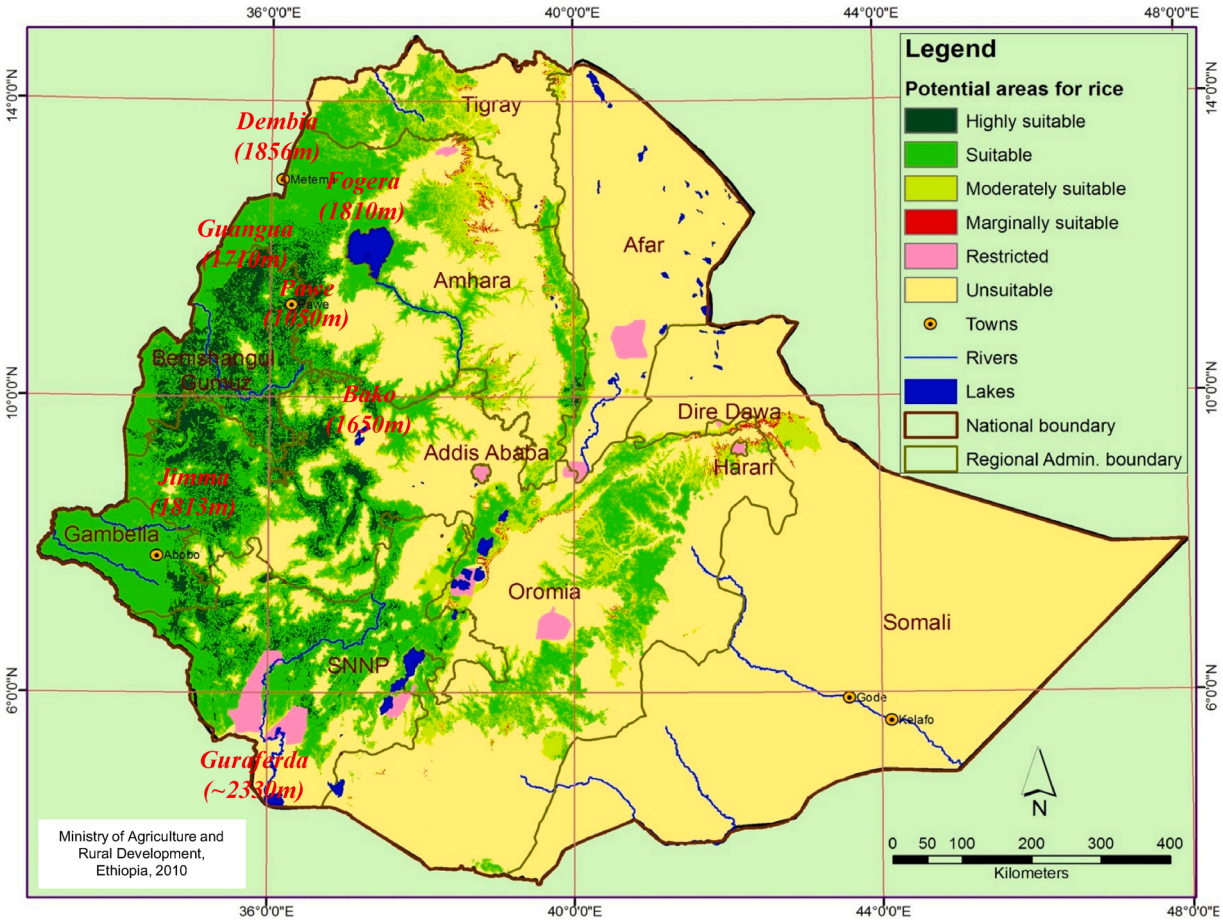

Fig. 1. Map of the rainfed rice production areas in Ethiopia and their suitability for rice production. Most of the national production (40 %) is grown in the Amhara region, followed by the Somali (19 %) and Southern Nation Nationalities and Peoples regions (10 %), and the Gambela region (11 %) (Ministry of Agriculture and Rural Development, Ethiopia, 2010). Boldface names indicate regions; italicized names represent areas within the regions with altitude.

fertilize) during flowering. Satake and Shibata (1992) tested 19 *japonica* cultivars and identified 'Akage' as the donor of a cold-tolerance gene related to factor 1, 'Hayayuki' as the donor of a gene related to factor 2, and 'Chubo 42' as the donor of a gene related to factor 3. Recently, Shimono et al. (2016) proposed a simple model based on anther length, which is proportional to the number of pollen grains that can develop (Satake, 1976; Satake and Shibata, 1992; Suzuki, 1982; Tanno et al., 1999), and measured length under cold stress and unstressed control conditions. Factors 1 and 2 were proportional to the anther length under control conditions, and to the ratio of anther length at low temperature to length under unstressed conditions, and identified the highly tolerant cultivar, 'Tohoku PL-3', as the donor of a gene related to factor 2 by means of quantitative trait locus analysis. Clarifying these factors in Ethiopian germplasm would help breeders to improve cold tolerance when they select cultivars that could donate a cold-tolerance gene for use in future breeding.

Here, we evaluated the genotypic variation in cold tolerance of Ethiopian germplasms using cold-water phenotyping facilities, and then characterized cold tolerance in relation to the male reproductive morphology, during 2 years of trials in Japan. Our goal was to identify whether easily measured morphological characteristics could be used to predict cold tolerance in Ethiopia without requiring the use of phenotyping facilities.

2. Materials and methods

2.1. Genetic material

We selected 18 accessions (Table 1) for the experiment from among those released in Ethiopia (Dessie, 2020). The 18 accessions were recommended to farmers, and included 'X-Jigna' (which originated in North Korea), which is the leading landrace in major rice growing areas of the Fogera and Amhara regions, in addition to the Tigray and Oromia regions. 'Shaga' was chosen for its high yield, and 'Wanzaye', 'Erib', and 'Abaye' were recently released for lowland ecosystems. 'Ediget' is an intermediate cultivar suitable for both upland and lowland ecosystems in Fogera. 'Pawe 1' and 'Chewaqa' are the major cultivars in the Benishangul Gumuz and Oromia regions, respectively. 'NERICA 4' is the major cultivar in upland ecosystems in all rice growing areas in Ethiopia. We used two standard Japanese check cultivars: 'Hitomebore', which has strong cold tolerance and is the major cultivar in northern Japan, and 'Sasanishiki', which has weak cold tolerance. Note that in Ethiopia, rice is ground to produce flour that is used as an alternative ingredient for a traditional Ethiopian staple food, *enjera*, which is traditionally composed of fermented teff (*Eragrostis tef*) flour. 'X-Jigna' and 'Shaga' are suitable for inclusion in *enjera* flour, even though they have comparable amylose contents to 'Ediget', which Ethiopian farmers consider unsuitable for use in *enjera*.

2.2. Treatments and experimental design

To test the cold tolerance of the 20 cultivars, we used two cold screening facilities, one with the plants grown in pots and the other with the plants grown under field conditions. Under both conditions, plants were exposed to cold water treatment throughout the reproductive development period.

2.2.1. Pot experiment in 2019

On 22 May 2019, we transplanted 26-day-old-seedlings of all cultivars into two 1/5000-a Wagner pots each (4 L pot, 16 cm in diameter, 20 cm in height), at 16 seedlings per pot. The experiment was conducted at Iwate University, in Morioka, Iwate, Japan (39°42′N, 141°8′E). The seedlings were planted in a circular arrangement, at a spacing of 3 cm between plants (Shimono et al., 2011). The pots were filled with commercial soil that contained 1.4 g N, 1.6 g P, and 1.5 g K (Agro-Baido fertilizer, Kanuma-Sangyo Co., Kanuma, Japan). To minimize the effects of phenological and physiological variation within a plant caused by differences in the number of tillers, we removed all tillers and retained

Table 1
Information on the 18 Ethiopian germplasms and 2 Japanese check cultivars used in this study.

Category	Cultivar	Year released	Where released	Ecosystem	Origin[a]	Subspecies [b]	Suitability for *enjera* flour[c]	Amylose content (%)[d]	Production in Ethiopia[a]
Ethiopian	Fogera 2	2016	Fogera	lowland	IRRI	*indica*	–	–	no
	Getachew	2007	Adet	upland	unknown	*indica*	–	–	no
	NERICA4	2006	Pawe	upland	Africa Rice Center	*japonica*	fair	21.2	yes
	Hiber	2011	Adet	lowland	Africa Rice Center	*japonica*	–	–	no
	X-jigna	2016	Fogera	lowland	North Korea	*japonica*	excellent	21.3	yes
	Fogera 1	2016	Fogera	upland	Africa Rice Center	*japonica*	–	–	no
	Chewaqa	2013	Bako	upland	China	*japonica*	good	–	yes
	Erib	2017	Fogera	lowland	Africa Rice Center	*japonica*	–	–	no
	Wanzaye	2017	Fogera	lowland	Madagascar	*japonica*	–	–	yes
	Abaye	2017	Fogera	upland	Africa Rice Center	*japonica*	–	–	no
	Superica-1	2006	Pawe	upland	Africa Rice Center	*japonica*	–	–	yes
	Pawe 1	1998	Pawe	upland	unknown	*japonica*	very good	15.2	yes
	Shaga	2017	Fogera	lowland	Madagascar	*japonica*	excellent	–	yes
	Gumara	1999	Adet	lowland	Africa Rice Center	*indica*	good	21.5	yes
	Adet	2014	Adet	upland	Africa Rice Center	*japonica*	fair	26.1	no
	Ediget	2011	Adet	lowland	Africa Rice Center	*japonica*	very poor	20.9	yes
	Tana	2007	Adet	upland	unknown	*indica*	–	–	no
	Andassa	2007	Adet	upland	unknown	*indica*	–	–	–
Japanese check	Sasanishiki	1963	–	lowland	Japan	*japonica*	–	14.4	no
	Hitomebore	1992	–	lowland	Japan	*japonica*	–	14.2	no

Sources: [a] IRRI, International Rice Research Institute, Dessie (2020); [b] Taddesse et al. (2020); [c] personal communication from farmers; [d] measured in rice grains harvested at Iwate University in 2019 (unpublished data).

only the main stem (Satake and Koike, 1983a). The plants were grown in a greenhouse under natural light. After the panicle initiation stage of each cultivar (with the young panicles about 1 mm long), we tested the cold tolerance of the plants in tubs (500 L, 122 cm × 76 cm, with a 54-cm depth) in which the water temperature (T_w) was controlled at 19.1 ± 0.4 °C (mean ± standard deviation) during the reproductive period, which lasted from 42 to 110 days after transplanting (DAT), of all cultivars (Supplemental Fig. S1). The heading date ranged from 77 DAT ('X-Jigna') to 106 DAT ('Andassa') (Supplemental Table S1). Water temperature was measured at 10-s intervals with Pt100 thermometer (R902-31, Chino Co., Tokyo, Japan) recoding on a datalogger (CR1000, Campbell Scientific, Loughborough, UK), and was controlled well by supplying cool water at ca. 12 °C (produced by a water cooling and circulation device (CA-400, Eyela, Tokyo, Japan) that circulated water through heat-exchange coils installed in the tubs via an electromagnetically controlled valve. The system uniformly exposed all panicles to low temperature. The water depth was kept at >30 cm above the shoot base to expose the developing panicles; these initiated at the shoot base and then lifted upward during growth (except during first 5 days after panicle initiation, when the water was kept at a depth of 15 cm to prevent excessive stem elongation). Water in the tubs was circulated by a high-flow air pump (C-5BN, Techno Takatsuki, Osaka, Japan) to minimize spatial variation in T_w. The treatments were maintained until the full flowering stage.

In the unstressed control conditions, another pot for each cultivar was grown after panicle initiation in a warm environment in the same greenhouse. The heading date of the earliest cultivar ('Ediget') was 69 DAT, and the latest cultivars ('Tana' and 'Andassa') at 99 DAT (Supplemental Table S1). The water depth was <5 cm; T_w was not controlled, and averaged 25.1 ± 0.4 °C (mean ± standard deviation), and T_a was 24.7 ± 2.5 °C during the reproductive period (Supplemental Fig. S1).

2.2.2. Field experiment in 2019

On 23 May 2019, seedlings of the same accessions used in the pot experiment were transplanted at a field phenotyping facility at the Iwate Prefecture Agricultural Research Center, in Kitakami, Iwate, Japan (39°35′N, 141°11′E), at 34.7 plants m^{-2} (0.24 m between rows, 0.12 m between plants, with one plant per spot). The facility can control T_w in a paddy field that is 9.5 m long in the direction from the water inlet to the outlet and 4.8 m wide (Supplemental Fig. S2). We planted 3 plants per accession at each of 3 locations (each location at 2.3-m intervals from the inlet to the outlet) in the cold treatment. Cold water was applied at 19.2 ± 0.5 °C (mean ± standard deviation) from 44 to 109 DAT; the water depth was kept at 20 cm until 48 DAT, and thereafter increased to 30 cm. The timing of start of cold treatment of 44 DAT is 33 days before the heading date of the earliest-heading accession ('X-Jigna', at 77 DAT) and the timing of end of the treatment of 109 DAT is 7 days before the latest cultivar's heading date ('Pawe 1', at 102 DAT) (Supplemental Table S1). Thus, the field experiment covered the reproductive development period of all accessions as well as it did in the pot experiment. Basal fertilizer was applied at 50 kg N ha^{-1}, 22 kg P ha^{-1}, and 42 kg K ha^{-1}.

Under the unstressed control conditions, seedlings of the same accessions were transplanted on 21 May 2019 at Iwate University. Nine plants of each cultivar were grown in a single row at 22.1 plants m^{-2} (0.30 m between rows, 0.15 m between plants, with one plant per spot). The heading date of the earliest cultivar ('X-jigna') was 71 DAT, and the latest cultivars ('Shaga') at 103 DAT (Supplemental Table S1). T_w in the control was 25.3 ± 2.1 °C, and T_a was 24.3 ± 3.1 °C during the reproductive period. Basal fertilizer was applied at 90 kg N ha^{-1}, 65 kg P ha^{-1}, and 75 kg K ha^{-1}.

2.2.3. Experiment in 2018

On 7 and 10 May 2018, 26- and 29-day-old-seedlings of all accessions were planted in pots and under field conditions, respectively, to evaluate the genotypic variation in reproductive morphology in a warm, unstressed environment at Iwate University. In the pot experiment, all cultivars were transplanted into two 1/5000-a Wagner pots filled with the same commercial soil used in the 2019 experiments, with four seedlings per pot (one plant per spot). The plants were grown either in tubs filled with water at ambient temperatures in a glasshouse or in a field environment: one pot in each environment. In the field experiment, 11 plants were grown at 22.1 hills m^{-2} (30 cm between rows, 15 cm between plants, at one plant per hill), which was equivalent to the unstressed field conditions in 2019. Water depth was kept at <5 cm throughout the growing season. Since the mean T_a and T_w during the period of reproductive development (50–120 DAT) were both >23.0 °C (Supplemental Fig. S3), and the effects of the treatment had a negligible effect on reproductive morphology, we present the mean value for the three conditions (i.e., inside and outside pots plus the field planting) in the following results.

2.3. Measurements

2.3.1. Days to heading

Days to heading was recorded in all experiments as when 50 % of the population in each cultivar had flowered.

2.3.2. Reproductive morphology

We measured the reproductive morphology on the day of anthesis or 1 day earlier. We collected 27 spikelets per accession (1 panicle × 9 spikelets × 3 plants) from the upper 3 primary branches of the rachis, at the 3rd, 4th, and 5th spikelets from the top position. Since the developmental stage among the spikelets in the single panicle differed by up to 7 days, we collected spikelets that had the same developmental stage by the criteria of Satake (1976). The collected spikelets were stored in 50 % ethanol until analysis in both years.

We measured the length and width of 48 anthers and 8 stigmas per accession in each treatment in 2018 and 24 anthers and 4 stigmas per accession in each treatment in 2019 from randomly chosen spikelets under a microscope (Model CHT, Olympus Optical Co., Ltd, Tokyo, Japan (a microscope with a 4× eyepiece and 10× primary lens). To determine pollen viability, we stained 12 anthers (2 spikelets × 6 anthers) from each accession in each treatment overnight in a mixture of 20 % Gram's stain reagent 2 (25 % glycerol, 25 % ethanol, and 30 % distilled water; Satake, 1976). From two randomly selected anthers per accession per treatment, we counted the total number of viable (stained black) pollen grains per anther under the microscope.

2.3.3. Spikelet fertility and plant heights

At harvest, we measured the culm length above the ground (the distance from the shoot base to the panicle base) in both years. We counted the total spikelet number per panicle and assessed spikelet fertility (percentage of total spikelets per panicle that were fertile) in 2019. Sterile spikelets were carefully identified by backlighting the heads with fluorescent light from a light box (Treviewer A3-400, Trytech Co., Tokyo, Japan); spikelets that showed no shadowy area (i.e., no developing embryo or grain) were considered to be sterile. Spikelet fertility was counted on all plants in pots (n = 14–16, except n = 7 for 'Chewaqa'), with only one panicle per plant because we removed all tillers before transplanting and retained only the main stem. Under field conditions, we measured 3 plants per cultivar in the cold treatment (top 3 panicles of each) at 3 locations (9 plants per accession), and 3 plants per accession in the control (top 3 panicles of each; 3 plants per accession).

2.4. Statistical analysis

We performed Peterson's linear regression analysis in Microsoft Excel 2019 (www.microsoft.com) to test the relationships between reproductive morphology and spikelet sterility under cold stress. Two-way ANOVA analysis was conducted using JMP version 14.2.0. (SAS

institute).

3. Results

Cold stress significantly reduced the spikelet fertility of the 18 Ethiopian rice germplasms, but the magnitude of the reduction differed among cultivars (Fig. 2); spikelet fertility under cold stress ranged from 9 % to 84 % in the pot experiment (Fig. 2a) and from 0 % to 85 % in the field experiment (Fig. 2b). The range of values of the Ethiopian germplasm overlapped the range of the Japanese cold-susceptible 'Sasanishiki' (7 % pot, 23 % field) and cold-tolerant 'Hitomebore' (82 % pot, 90 % field). The effects of genotype (G) and treatment (T), and their interactions on spikelet fertility were significant for both pot and field environments ($P < 0.001$).

The variation in spikelet fertility in pots was significantly and moderately strongly correlated with the results in the field experiment (Pearson's $r = 0.70$, $P < 0.001$). From the mean values from the two phenotyping results (Fig. 2c), we categorized the cultivars into 6 ranks (at 16 % intervals) based on spikelet fertility, with higher numbers representing higher tolerance to cold stress. 'Andassa' and 'Tana' had the highest fertility (rank 5, tolerant) and 'Fogera 2' and 'Getachew' had the lowest (rank 1, susceptible) (Table 3). 'Hitomebore' was rank 6 and 'Sasanishiki' was rank 1.

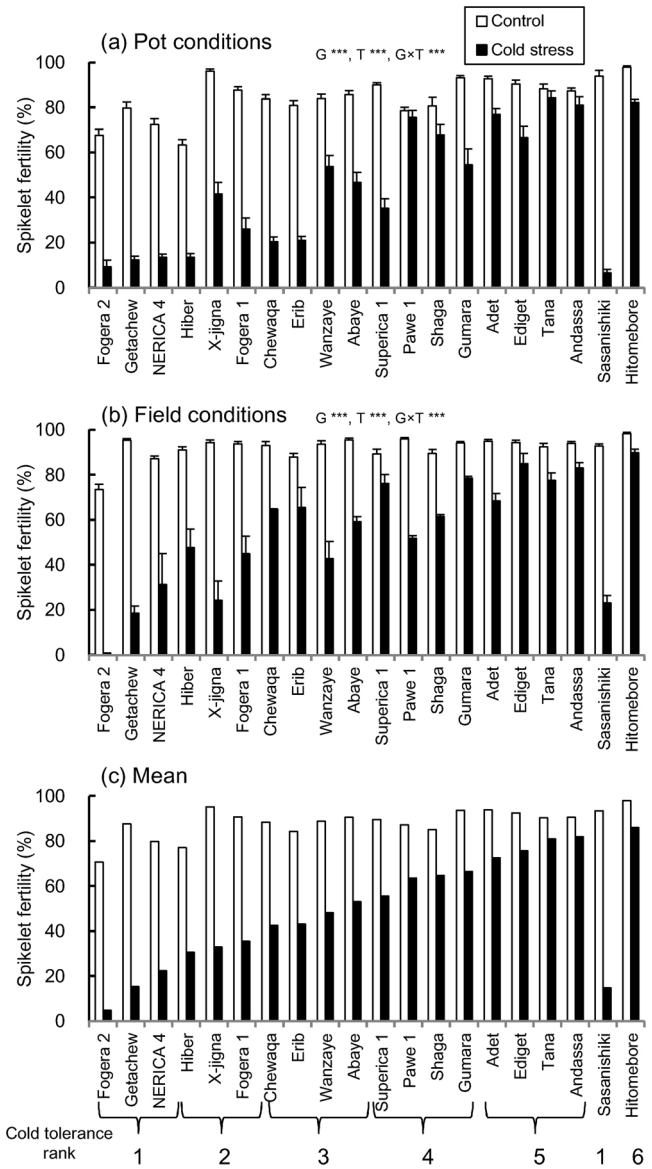

Fig. 2. Spikelet fertility of the 18 Ethiopian cultivars and 2 Japanese check cultivars exposed to cold during reproductive development in 2019: (a) pot experiment, (b) field experiment, and (c) mean of the two experiments. Values are mean ± standard error. In the pot experiment, $n = 14$ to 16 except $n = 7$ for 'Chewaqa' in the cold stress treatment, and $n = 8$ to 9 except $n = 3$ for 'Sasanishiki' in the control. In the field experiment, $n = 9$ in the cold stress and $n = 3$ in the control, except $n = 1$ for 'Chewaqa' in the control. ANOVA : genotype (G), treatment (T), and G × T, ***, $P < 0.001$.

The variation in spikelet fertility under cold stress in the pot experiment was explained well by anther length ($r = 0.81$, $P < 0.001$), followed by fertile pollen number ($r = 0.70$, $P < 0.001$), and anther width ($r = 0.69$, $P < 0.001$) (Table 2). A 0.1-mm decrease of anther length caused a 6.8 percentage point decrease of spikelet fertility (Fig. 3a) and a 145-grain decrease in the number of viable pollen grains per anther (Fig. 3b). We found no significant relationship between spikelet fertility and stigma length, but the stigma width was marginally positively correlated with spikelet fertility ($r = 0.42$, $P < 0.10$) (Table 2).

Anther length ranged from 1.62 to 2.50 mm in the control and from 0.93 to 1.74 mm under cold stress, and the number of fertile pollen grains per anther ranged from 796 to 2441 in the control and from 2 to 1440 under cold stress (Table 2). Interestingly, the anther length and the number of fertile grains in the control, with normal spikelet fertility (up to 98 %; Fig. 1), were significantly or marginally significantly positively correlated with spikelet fertility under cold stress ($r = 0.66$ for anther length, $P < 0.01$; $r = 0.41$ for fertile pollen, $P < 0.10$; Table 2, Fig. 4). Even in the unstressed control in 2018, anther length varied among cultivars, ranging from 1.80 to 2.44 mm (Table 2), and it was moderately strongly correlated with spikelet fertility under cold stress in 2018 ($r = 0.64$ in pots, $r = 0.58$ in the field, $r = 0.66$ for the mean, all $P < 0.01$), as it was in 2019 (Fig. 4), suggesting that anther length measured under unstressed conditions may predict a cultivar's cold tolerance.

4. Discussion

We found high genotypic variation in cold tolerance among the 18 Ethiopian cultivars, with spikelet fertility ranging from 0% to 90 % (Fig. 2). This result appears to be reliable because the rank of spikelet fertility under cold stress was consistent in all phenotyping methods ($r = 0.70$, $P < 0.01$). In addition, the Japanese tolerant cultivar 'Hitomebore' and the susceptible 'Sasanishiki' covered the range between highest and lowest spikelet fertilities at both sites (Fig. 2c, Table 3), agreed with previous rankings of these cultivars (Koumoto et al., 2016; Shimono et al., 2011). This phenotyping successfully divided the Ethiopian germplasm into six cold tolerance ranks, which will be useful to support future screening of new cultivars by providing standard reference cultivars (i.e., benchmarks), and will therefore support future breeding for cold tolerance.

We identified two candidate cultivars that could serve as donors of cold tolerance genes: 'Andassa' and 'Tana' both had rank 5 (tolerant), and were comparable to the high-tolerance cultivar 'Hitomebore', with rank 6 (Fig. 2c, Table 3). These two cultivars were released in 2007 by the Adet Research Center (2240 m a.s.l.) for use in upland rice ecosystems (Dessie, 2020). Recently, Taddesse et al. (2020) genotyped 79 Ethiopian cultivars (including our 18 cultivars) using 50 molecular markers, and categorized them as *indica* recombinants (Table 1), although their source is unknown. In the terms of the three physiological mechanisms involved during the fertilization processes (Satake and Shibata, 1992; Shimono et al., 2016), 'Andassa' and 'Tana' had a 6 %–10 % higher ratio of anther length (0.67–0.69) under cold stress to the length in the control, which was greater than the means for all cultivars combined (Table 2). This suggests higher tolerance of pollen and anthers to cold episodes based on factor 2. Note that, since cold tolerance was tested not only at the booting stage which is the most sensitive stage (Satake, 1976), but also during the early reproductive stage of pollen development using cold water >30 cm after panicle initiation, the tolerance of factor 2 included the processes of pollen development.

Table 2
Reproductive morphology of the 18 Ethiopian germplasms and 2 Japanese check cultivars in the control and cold stress treatments during the reproductive development period, their correlation (Pearson's *r*) with spikelet fertility in the pot and field experiments, and the mean values in the two experiments.

Genotype	Experiment 1. pot (2019)																		Experiment 2. (2018)					
	Anther								Stigma								Viable pollen number/ anther		Anther				Stigma	
	Length (mm)					Width (mm)			Length (mm)				Width (mm)						Length (mm)		Width (mm)		Length (mm)		Width (mm)	
	CT		Cold		Ratio	CT		Cold		CT		Cold		CT		Cold		CT	Cold		CT		CT		CT	
Fogera 2	2.14	±0.01	1.30	±0.02	0.61	0.45	±0.02	0.33	±0.01	1.09	±0.03	0.88	±0.04	0.27	±0.01	0.16	±0.01	1372 ±60	71 ±36	2.10	±0.03	0.43	±0.02	1.07	±0.04	0.31	±0.01
Getachew	1.89	±0.02	0.93	±0.02	0.49	0.41	±0.01	0.33	±0.01	0.62	±0.02	0.52	±0.04	0.29	±0.01	0.17	±0.00	1468 ±127	14 ±13	1.91	±0.02	0.42	±0.00	0.62	±0.02	0.25	±0.00
NERICA4	2.09	±0.03	1.15	±0.04	0.55	0.41	±0.00	0.36	±0.01	0.76	±0.02	0.68	±0.04	0.31	±0.02	0.27	±0.00	2038 ±90	221 ±36	2.25	±0.01	0.43	±0.01	0.75	±0.04	0.30	±0.02
Hiber	2.01	±0.03	1.29	±0.03	0.64	0.42	±0.00	0.34	±0.01	0.67	±0.03	0.68	±0.02	0.28	±0.03	0.20	±0.02	1753 ±74	122 ±39	2.19	±0.03	0.44	±0.00	0.77	±0.01	0.31	±0.01
X-jigna	1.95	±0.02	1.13	±0.04	0.58	0.41	±0.01	0.36	±0.01	0.79	±0.02	0.69	±0.07	0.32	±0.02	0.26	±0.01	1463 ±64	276 ±3	1.80	±0.02	0.42	±0.01	0.71	±0.02	0.31	±0.00
Fogera 1	2.12	±0.03	1.18	±0.03	0.56	0.43	±0.01	0.36	±0.01	0.87	±0.03	0.80	±0.03	0.35	±0.02	0.27	±0.03	1745 ±21	174 ±121	2.06	±0.03	0.41	±0.00	0.81	±0.03	0.33	±0.01
Chewaqa	1.98	±0.02	1.23	±0.02	0.62	0.43	±0.00	0.34	±0.01	1.19	±0.04	1.29	±0.02	0.48	±0.01	0.40	±0.02	1205 ±65	21 ±7	1.87	±0.03	0.41	±0.00	1.04	±0.02	0.36	±0.01
Erib	1.98	±0.02	1.24	±0.03	0.63	0.42	±0.01	0.38	±0.01	0.82	±0.03	0.87	±0.05	0.39	±0.03	0.33	±0.05	1779 ±19	554 ±73	2.12	±0.03	0.43	±0.00	0.87	±0.04	0.33	±0.01
Wanzaye	2.24	±0.02	1.58	±0.03	0.70	0.61	±0.03	0.39	±0.01	0.68	±0.07	0.66	±0.05	0.30	±0.04	0.29	±0.03	2274 ±39	844 ±196	2.17	±0.00	0.41	±0.01	0.67	±0.03	0.27	±0.02
Abaye	2.15	±0.02	1.39	±0.02	0.65	0.44	±0.01	0.38	±0.01	1.17	±0.02	1.13	±0.03	0.39	±0.04	0.26	±0.01	1669 ±67	187 ±54	2.17	±0.02	0.42	±0.00	1.19	±0.04	0.38	±0.01
Superica-1	2.28	±0.02	1.31	±0.03	0.57	0.54	±0.02	0.39	±0.01	0.79	±0.06	0.78	±0.02	0.37	±0.03	0.30	±0.01	2140 ±74	113 ±48	2.27	±0.01	0.49	±0.02	0.80	±0.01	0.37	±0.01
Pawe 1	2.50	±0.02	1.45	±0.02	0.58	0.46	±0.01	0.39	±0.00	1.02	±0.03	0.82	±0.04	0.39	±0.01	0.33	±0.02	2075 ±9	417 ±122	2.43	±0.01	0.47	±0.00	0.97	±0.03	0.41	±0.01
Shaga	2.20	±0.02	1.74	±0.01	0.79	0.44	±0.01	0.42	±0.01	0.84	±0.04	0.82	±0.03	0.40	±0.04	0.42	±0.02	2441 ±128	1440 ±81	2.16	±0.01	0.44	±0.02	0.75	±0.02	0.37	±0.02
Gumara	2.24	±0.02	1.37	±0.02	0.61	0.43	±0.02	0.35	±0.01	0.99	±0.03	1.20	±0.02	0.43	±0.02	0.33	±0.02	1594 ±103	532 ±35	2.35	±0.02	0.43	±0.01	1.02	±0.04	0.38	±0.01
Adet	2.29	±0.01	1.58	±0.04	0.69	0.37	±0.01	0.37	±0.01	1.04	±0.00	0.95	±0.06	0.37	±0.02	0.28	±0.01	1616 ±66	785 ±26	2.39	±0.00	0.40	±0.00	0.96	±0.04	0.35	±0.01
Ediget	2.12	±0.02	1.39	±0.02	0.65	0.48	±0.01	0.40	±0.01	0.77	±0.01	0.74	±0.04	0.40	±0.01	0.35	±0.02	1749 ±100	709 ±115	2.30	±0.03	0.47	±0.01	0.65	±0.00	0.34	±0.01
Tana	2.35	±0.02	1.57	±0.03	0.67	0.37	±0.00	0.36	±0.00	1.03	±0.02	1.01	±0.05	0.34	±0.02	0.29	±0.02	1917 ±39	658 ±32	2.33	±0.02	0.40	±0.01	1.00	±0.04	0.36	±0.01
Andassa	2.40	±0.02	1.66	±0.04	0.69	0.37	±0.00	0.38	±0.00	0.96	±0.06	0.93	±0.07	0.36	±0.03	0.27	±0.03	2041 ±134	837 ±38	2.44	±0.02	0.41	±0.01	1.04	±0.04	0.37	±0.01
Sasanishiki	1.62	±0.02	0.98	±0.03	0.61	0.38	±0.01	0.30	±0.01	0.91	±0.01	0.94	±0.04	0.43	±0.01	0.37	±0.02	796 ±1	2 ±1	1.82	±0.03	0.40	±0.00	0.86	±0.01	0.39	±0.01
Hitomebore	1.97	±0.01	1.41	±0.02	0.72	0.40	±0.00	0.40	±0.01	0.96	±0.05	1.10	±0.05	0.47	±0.04	0.53	±0.03	1328 ±32	346 ±29	2.13	±0.01	0.45	±0.00	0.91	±0.04	0.47	±0.02
Max	2.50		1.74		0.79	0.61		0.42		1.19		1.29		0.48		0.53		2441	1440	2.44		0.49		1.19		0.47	
Min	1.62		0.93		0.49	0.37		0.30		0.62		0.52		0.27		0.16		796	2	1.80		0.40		0.62		0.25	
Mean	2.13		1.35		0.63	0.43		0.37		0.90		0.88		0.37		0.30		1723	416	2.16		0.43		0.87		0.35	
CV%	10		16		11	13		8		18		23		16		28		23	90	9		6		18		14	
Correlation coefficient																										
(a) FT in cold ain pot	*0.66* **		*0.81* ***		*0.63* **	*−0.06* *ns*		*0.69* ***		*0.27* *ns*		*0.27* *ns*		*0.31* *ns*		*0.42* +		*0.41* +	*0.70* ***	*0.64* **		*0.08* *ns*		*0.21* *ns*		*0.51* *	
(b) FT in cold in field	*0.43* +		*0.58* **		*0.51* *	*−0.04* *ns*		*0.61* **		*0.23* *ns*		*0.48* *		*0.60* **		*0.59* **		*0.29* *ns*	*0.46* *	*0.58* **		*0.23* *ns*		*0.23* *ns*		*0.61* **	
(c) FT of mean of pot and field	*0.60* **		*0.76* ***		*0.62* **	*−0.05* *ns*		*0.71* ***		*0.27* *ns*		*0.40* +		*0.49* *		*0.54* **		*0.38* +	*0.64* ***	*0.66* **		*0.16* *ns*		*0.24* *ns*		*0.60* **	
Genotype (G)	***					***				***				***				***		***		***		***		***	
Treatment (T)	***					***				+				***				***		–		–		–		–	
G x T	***					***				***				*				***		–		–		–		–	

CV, coefficient of variation; FT, spikelet fertility. Correlation coefficient (Pearson's r) of the relationship between each morphological parameter and spikelet fertility under cold stress in (a) pot experiment and (b) field experiment, and (c) mean values of the two experiments. Significance: ***P < 0.001, **P < 0.01, *P < 0.05, +P < 0.10; ns, not significant. Values are mean ± standard error (n = 20–24 for anthers, n = 4 for stigmas, n = 2 for fertile pollen number in 2019, and n = 3 in 2018).

Table 3
Cold tolerance rank based on the spikelet fertility of the 18 Ethiopian germplasms and 2 Japanese check cultivars with cold stress imposed during reproductive development in the pot and field experiments. Cold tolerance ranks were defined in 16 % fertility intervals from 1 (susceptible) to 6 (tolerant).

Accession	Spikelet fertility (%)			Cold tolerance rank	
	Pot	Field	Mean		
Fogera 2	9	0	5	1	Susceptible
Getachew	12	19	16	1	Susceptible
NERICA4	14	31	22	1	Susceptible
Hiber	14	48	31	2	Moderately susceptible
X-jigna	42	24	33	2	Moderately susceptible
Fogera 1	26	45	36	2	Moderately susceptible
Chewaqa	20	65	43	3	Moderate
Erib	21	66	43	3	Moderate
Wanzaye	54	43	48	3	Moderate
Abaye	47	59	53	3	Moderate
Superica-1	35	76	56	4	Moderate tolerant
Pawe 1	76	52	64	4	Moderate tolerant
Shaga	68	62	65	4	Moderate tolerant
Gumara	55	79	67	4	Moderate tolerant
Adet	77	68	73	5	Tolerant
Ediget	67	85	76	5	Tolerant
Tana	84	78	81	5	Tolerant
Andassa	81	83	82	5	Tolerant
Sasanishiki	7	23	15	1	Very susceptible
Hitomebore	82	90	86	6	Very tolerant
Max	84	90	86	6	
Min	7	0	5	1	
Mean	45	55	50	3	
CV (%)[a]	63	46	49	49	

[a] CV, coefficient of variation.

In addition, 'Andassa' and 'Tana' had a 10 % longer anther length (2.35–2.40 mm) than the mean value in the control and an 11 %–14 % greater number of viable pollen grains (1917–2041) than in the control (Table 2). This suggests a greater ability to initiate pollen grain development in the anther during early reproductive development to increase the potential number of pollen grains, which we described in the Introduction as factor 1. Thus, 'Andassa' and 'Tana' had strong cold tolerance due to both factor 1 and factor 2, and these cultivars would therefore be useful genetic donors for future breeding in Ethiopia; in Kenya, a highland in central Africa; in Senegal, in western Africa; and in Madagascar, where rice is grown at high latitudes. These areas cover an estimated 7 % of the total area of Africa that is affected by cold (van Oort, 2018). Interestingly, Japanese cold-tolerant check 'Hitomebore' showed tolerance based on only factor 2; a 16 % higher ratio of anther length (0.72) under cold stress to the length in the control than the mean value (Table 2); not factor1, a 8 % shorter anther length (1.97 mm) than the mean value in the control. This suggests that the two Ethiopian cultivars, 'Andassa' and 'Tana', might have potential to improve cold tolerance of 'Hitomebore', granted the ability initiating greater number of pollen grains.

On the other hand, the current leading cultivars in Ethiopia, 'X-Jigna' and 'NERICA 4', which were developed for lowland and upland ecosystems, respectively, had a cold tolerance rank of 2 and 1, respectively, which is weak (Fig. 1c, Table 3). To extend the cultivated area and the cultivation season while reducing the risk of cold damage, it would be useful to introduce genes from 'Andassa' and 'Tana' and other cold-tolerant resources such as 'Hitomebore' into new cultivars.

Our screening method used cold water at >30 cm depth, and we exposed the developing panicles to low temperatures during the period from panicle initiation around the young microspore stage which is the most sensitive stage (Satake et al., 1988). Kobayashi and Satake (1979) monitored the vertical positions of the individual spikelets of five cultivars at different N fertilization during stem elongation, and showed that panicle position at the young microspore stage was ranged from 16~21 cm. Our treatment of water depth at >30 cm would cover the most sensitive reproductive stage of all genotypes. On the other hand, after the panicles emerged above the water level such as the flowering stage, the method did not control the panicle temperature. We might overlook the genotypic variation in cold tolerance at flowering stage (Satake and Koike, 1983a, 1983b). Additionally, there was genotypic variation in heading date for the cultivars tested (Supplemental Table S1), and this can change the duration of the exposure of the developing panicles to cold treatment relative to the total reproductive phase in each cultivar, which might induce errors in the evaluation of cold tolerance rank. Further confirmation studies using different method such as controlling air temperature in growth cabinets (Farrell et al., 2006) and on-site multi-environmental field trials combined with model analysis (Shimono et al., 2007) would be required.

In Ethiopia, where cold screening facilities are currently not available, field trials in areas with different environmental conditions are currently the only option to support breeding programs. Here we defined the cold tolerance ranks for 18 Ethiopian cultivars in different maturity groups (Fig. 2c, Table 3), which can now be used as standard

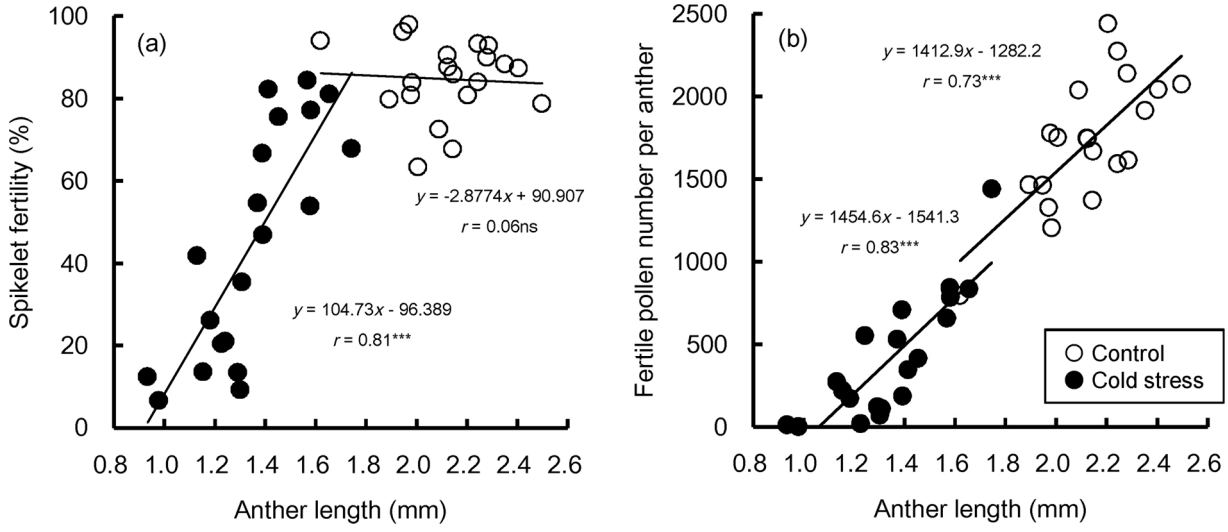

Fig. 3. Relationships between (a) anther length and spikelet fertility, and (b) anther length and the number of fertile pollen grains per anther in the 18 Ethiopian cultivars and 2 Japanese check cultivars after exposure to cold stress during reproductive development. Significance: ***$P < 0.01$, ns not significant.

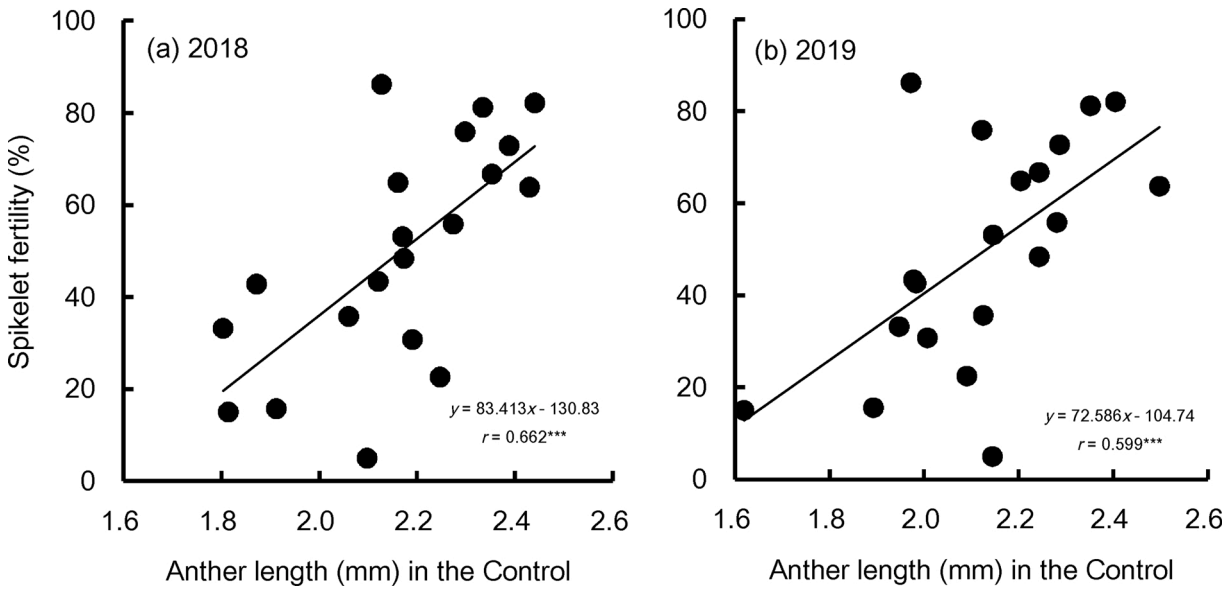

Fig. 4. Relationship between spikelet fertility under cold stress during reproductive development and anther length in the unstressed control in (a) 2018 and (b) 2019 in the 18 Ethiopian cultivars and 2 Japanese check cultivars. Significance: ***$P < 0.01$.

check cultivars for cold tolerance without controlling the field environment by considering their maturity group. We also found a close correlation between spikelet fertility and anther length under unstressed control conditions in different years and under different conditions (Fig. 4). This agrees with previous studies (Suzuki, 1982; Tanno et al., 1999). This means that the anther length under unstressed conditions, such as in a greenhouse, will be a useful criterion for screening for cold tolerance. The cold-tolerant 'Andassa' and 'Tana' had longer anthers, whereas the susceptible 'Getachew' had shorter anthers (Table 2). However, it should be noted that the physiological mechanism for fertilization under cold conditions is determined by several factors (Satake and Shibata, 1992; Shimono et al., 2016; Susanti et al., 2019). For example, 'Fogera 2' had long anthers (Table 2) but very weak cold tolerance (Table 3), which may cause lower survival rate of the pollen after exposure to cold (Shimono et al., 2016) and/or difficult anther dehiscence shed pollen on stigma (Susanti et al., 2019). Thus, anther length will be a useful pre-screening criterion for cold-tolerant germplasm, but it should be combined with field trials under a range of environmental conditions to confirm tolerance.

5. Conclusion

We found high genotypic variation in cold tolerance of 18 Ethiopian cultivars. We found that anther length under unstressed control conditions provides a potential proxy for pollen viability, possibly eliminating the need for dedicated phenotyping facilities. The results of our analysis reveal promising candidate donors of cold-tolerance genes for use in future breeding, and provide a standard set of check cultivars that can support future breeding.

CRediT authorship contribution statement

Habtamu Assega Alemayehu: Writing - original draft, Data curation. **Gibrilla Dumbuya:** Data curation. **Mehedi Hasan:** Data curation. **Tilahun Tadesse:** Validation. **Shinsuke Nakajyo:** Data curation, Methodology. **Tomoaki Fujioka:** Data curation, Methodology. **Akira Abe:** Data curation, Methodology. **Maya Matsunami:** Writing - review & editing. **Hiroyuki Shimono:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fcr.2020.108042.

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