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An efficient method to produce segregating populations in quinoa (*Chenopodium quinoa*)

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Abstract

Quinoa offers a promising alternative for staple food, considering its outstanding nutritional value and tolerance to abiotic stresses. To develop breeding programmes in quinoa, a reliable crossing method for increasing the genetic variation is required. In the following study, we aimed to develop segregating populations in quinoa. We tested the efficiency of three different crossing methods (hand emasculation, warm water emasculation and no emasculation). Moreover we developed a two-stage selection strategy based on morphological traits and molecular markers for the selection of hybrid plants. We reported hand emasculation to be the most efficient crossing method, followed by warm water emasculation and no emasculation. Our results demonstrated that crosses in quinoa can be successfully performed, despite its complicated flower structure and high self-pollination rate. Additionally, we developed 30 segregating populations from crosses between accessions of different origins with varying phylogenetic relationship, which offers a promising perspective for quinoa breeding programmes in the future.

KEYWORDS
genetic diversity, hand emasculation, molecular markers, plant breeding, self-pollinating crops, warm water emasculation

1 | INTRODUCTION

Rapid global population growth together with the lack of availability of arable land and accessible water is imposing a challenge especially in many poor countries, to feed the population with sufficient and nutritious food. The pseudocereal quinoa, a member of the Amaranthaceae family, has gained increasing interest as an alternative staple food particularly in marginal lands, due to its high nutritional value and strong tolerance to abiotic stresses like drought, salinity, frost and heat (Jacobsen et al., 2003). Quinoa originated and has been cultivated along the Andes region of South America for the last 7,000 years (Williams & Brenner, 1995). Quinoa seeds have a high protein level (10 to 18%) with a perfect balance of amino acids specifically the essential amino acids (Vilche et al., 2003). They also provide a valuable combination of beneficial micronutrients like potassium, copper, zinc, iron and calcium along with fibre, lipids, carbohydrates and vitamins (Vega-Gálvez et al., 2010). Moreover quinoa is low in gluten, which offers a perfect substitute for wheat for people suffering from celiac disease.

Despite of its exceptional characteristics, the seed yield of quinoa is generally low (around 1–2 t/ha), which makes breeding activities inevitable. Activities to breed varieties with higher seed yield

and good nutritional quality were driven from a small number of accessions in the Altiplano, which constitutes a very narrow genetic base for quinoa breeding programmes (Jacobsen & Mujica, 2002). Therefore, along with the conservation of landraces, which is essential for the preservation of the genetic material, efforts should be concentrated on the introduction of new germplasm into breeding programmes to increase genetic diversity. Quinoa is mainly an autogamous species with very small flowers, which particularly complicates crosses. Therefore, development of an efficient crossing method should serve as the first step in quinoa breeding programmes.

Quinoa produces panicle inflorescences consisting of mostly hermaphrodite, but also pistillate flowers. The hermaphrodite flowers consist of five sepals and five stamens surrounding the ovary, and two stigmas (Abdelbar, 2018; Figure 1a). The hand emasculation of quinoa flowers has shown to be very difficult due to the floral morphology, the size of the flowers and the rapid progress of flowering within the inflorescence (Jacobsen & Stølen, 1993; Peterson et al., 2015). In many species, heat treatment and vacuum emasculation have been routinely applied as alternative methods for physical emasculation of the seed parents (Almeida et al., 2017; Mukasa et al., 2007; Otsuka et al., 2010; Sha, 2013). Warm water emasculation relies on the fact that pollen is generally more sensitive to higher temperatures than the ovary and the stigma. Therefore, selecting the appropriate temperature, at which the pollen will not be viable anymore, but the ovary and stigma are still active (usually around 45°C), is crucial for the application of this method (Sha, 2013). Almeida et al. (2017) reported the hyperthermotherapy in a water bath of 46°C for a panicle immersion time of 2.5 min to be more effective for the production of sterile plants compared to vacuum emasculation in rice. However, warm water emasculation has been reported as ineffective in quinoa, because it damages the inflorescence (Fleming & Galwey, 1995). Stetter et al. (2016) considered three different crossing methods (open pollination, warm water emasculation and hand emasculation) for creating inter- and intra-specific hybrids in three grain species of the genus Amaranth. Their results indicated hand emasculation and open pollination to be the most and the least efficient methods for creating hybrids, respectively.

As an alternative to mechanical emasculation, male sterility systems can be considered as the method of choice, particularly for the production of hybrid varieties in a commercial level. Different sources for male sterility have been identified in quinoa germplasm (Simmonds, 1971; Ward & Johnson, 1994), including a cytoplasmic male sterility system, for which restorer genes can be found in many quinoa accessions (Ward, 1998). However, introducing this CMS system into parents to facilitate crossings is time-consuming. Moreover using the CMS system in quinoa breeding programmes does not seem to be feasible in the near future, since heterosis has not been reported yet and therefore commercial hybrid production seems elusive at the moment. Breeding inbred line varieties is still the method of choice as the floral morphology of quinoa rather promotes line development.

Apart from the availability of efficient methods for crossing, strategies for the identification of F_1 plants are also required to produce a large array of F_2 populations. Two strategies for the identification of F_1 plants have been proposed using morphological and/or molecular markers. In case of the former, parents should differ by easy to detect qualitative morphological traits with dominance/recessive inheritance. In quinoa, seed colour, inflorescence color, axillary pigmentation, and plant colour could be used as morphological markers, if the pollinator is homozygous for the dominant alleles (Peterson et al., 2015). This approach would however limit
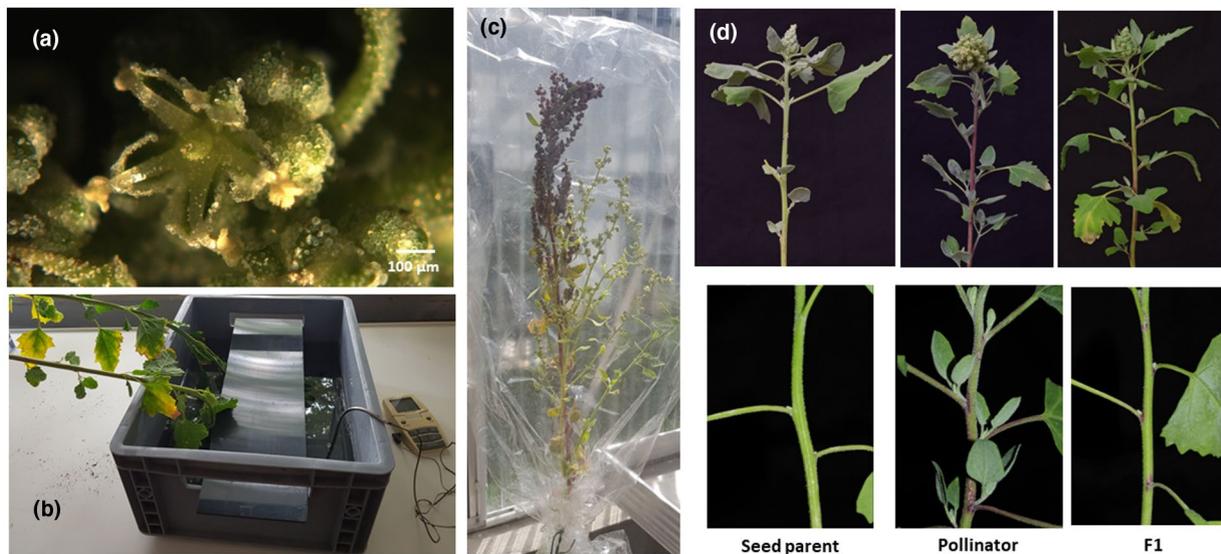

FIGURE 1 Floral morphology, crossing methods and selection of hybrid plants. (a) A hermaphrodite flower during pollination (stage BBCH60). The stamen consists of five anthers and the filaments form a ring around the ovary of the carpel. (b) Warm water emasculation crossing method. (c) Growing seed parent and pollinator side by side under a plastic bag ('no emasculation method'). (d) Axil pigmentation as morphological marker for selection of F_1 plants. Seed parents with green axil pigmentation were crossed with red-axil pigmented pollinators. The F_1 plants showed red axil pigmentation. Photos were taken at BBCH59

the number of crosses that can be performed, because only parents with contrasting phenotypes can be used. On the contrary, selection with molecular markers would allow crosses between any two parents, even if they show exactly the same morphological traits. Theoretically, one marker that is polymorphic between the two parents would be enough for the selection of true F_1 plants.

In the current study, we compared three different crossing methods to produce F_1 seeds. We found that one polymorphic marker locus unequivocally distinguishes F_1 hybrid plants from selfing offspring. In combination with phenotypic selection, we propose a two-step selection procedure for the identification of true F_1 plants. Moreover we produced 30 different F_2 populations, which can serve as starting material in quinoa breeding programmes.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

To test different crossing methods and their efficiency in quinoa (*Chenopodium quinoa* Willd.), we considered 12 red-axil and 11 green-axil quinoa accessions from five different countries of origin

TABLE 1 Quinoa accessions, which were used as crossing parents in this study. Sequencing data were available for all accessions except the ones marked by[a]

Seed code	Accession name	Geographical origin	Axil pigmentation
170875[a]	CHEN-104	Bolivia	Red
170876	CHEN-109	Peru	Red
170916	CHEN-160	Bolivia	Red
170985[a]	CHEN-340	Peru	Red
171008	CHEN-474	Peru	Red
171139	PI-634920	Chile	Red
171198	Ollague	Chile	Red
171205	Pasankalla	Peru	Red
171519	CHEN-119	Bolivia	Red
171599	CHEN-481	Peru	Red
180322	D-12166	Bolivia	Red
180450	Kamiri	Bolivia	Red
170867	PI-614886	Chile	Green
170886	CHEN-124	Bolivia	Green
171010	Ames-13743	Chile	Green
171024	PI-433232	Chile	Green
171079	PI-614883	Argentina	Green
171115	PI-614889	Chile	Green
171169	CHEN-69	Peru	Green
171214[a]	Real	Bolivia	Green
171230	Titicaca	Denmark	Green
171510	CHEN-83	Peru	Green
171605	PI-587173	Argentina	Green

for crossing (Table 1). Accessions with the dominant morphological trait (red axil) were considered as male parent and accessions with the recessive morphological trait (green axil) were considered as female parents for the crossing experiment (Table 1). We planted two plants per accession in 13 cm^2 pots in a cold greenhouse in May 2018 under natural long-day conditions in Kiel, Germany. To synchronize the flowering time of both crossing parents, we sowed the late-flowering accessions in the first week and then considered five different sowing dates in five consecutive weeks for the early-flowering accessions, based on the flowering time data of all the accessions from previous experiments (data not shown). We harvested the seeds on the seed parent and sowed 280 seeds per cross in 35x-multi trays in September 2018 in the cold greenhouse to identify the hybrid progenies. After leaf sampling for DNA isolation, we transferred the putative F_1 plants to 13 cm^2 pots for efficient seed production. All the true F_1 plants were bag-isolated to produce F_2 seeds.

2.2 | Crossing methods

We considered three different crossing methods for the production of F_1 seeds (Figure 1b,c and Figure 2).

2.2.1 | Hand emasculation of the seed parent

For this method, we followed the procedure suggested by Peterson et al. (2015) with slight modifications. To reduce the number of flower clusters to a manageable number, the flower bud of the seed parent was removed with scissors, once it was visible on the top of the plant (approximately 1.5 cm in size). We kept only 3–4 flower clusters on the seed parent. Then we opened the flowers under a magnifying lens (with 10x magnification) and removed all five anthers with a tweezer, when they were still green or yellowish-green (Figure 2).

2.2.2 | Emasculation of the seed parent with warm water

The inflorescence of the seed parents was dipped into a water bath of 45°C for 10 min (Figure 1b). This procedure was previously reported to be successful for the emasculation of the seed parents in grain amaranth (Stetter et al., 2016).

2.2.3 | No emasculation

Here we did not perform any treatments for the emasculation of the seed parent and simply placed the seed parent and the pollinator together under an isolation bag (Figure 1c).

Independent of the crossing method, we selected the pollinator plants at early stages of anthesis for crosses, where we expected a
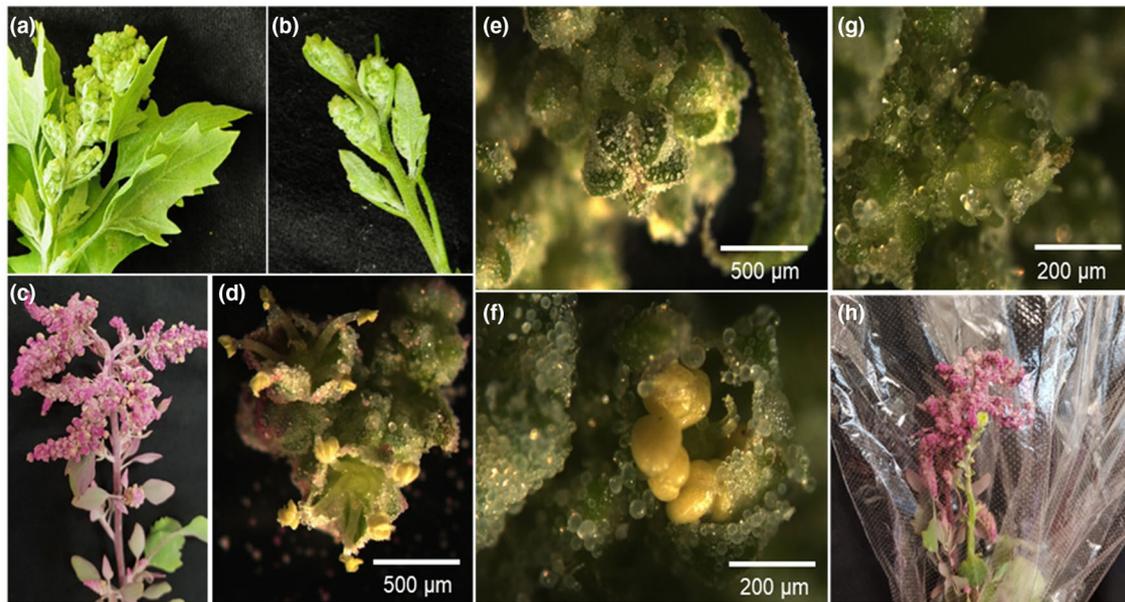
FIGURE 2 Hand crossing procedure in quinoa. (a) Seed parent short before flowering stage, (b) seed parent ready for hand emasculation, (C) pollinator with open flowers, (d) pollinator flowers at crossing stage, (e) seed parent flowers at crossing stage, (f) seed parent flowers were opened using a tweezer and the first anther was removed, (g) all 5 anthers were removed from the seed parent flower, (h) seed parent and pollinator were fixed to each other and were covered with an isolation bag

higher chance of successful fertilization over the course of several days. Then the seed parent and the pollinator were placed under an isolation bag and we shook the bags every day to accelerate the fertilization efficiency.

2.3 | DNA extraction

We collected leaf samples from all the putative F_1 plants and lyophilized them before DNA isolation. We used the NucleoSpin® Plant II DNA isolation kit (Macherey-Nagel) or CTAB method with slight modifications (Saghai-Maroof et al., 1984) to extract the DNA from the dried leaf samples.

2.4 | Confirmation of crosses

We conducted a two-stage selection for the identification of F_1 plants. First, we selected all the putative F_1 plants based on axil pigmentation. We phenotyped the seedlings for axil pigmentation four weeks after sowing. The putative F_1 plants are the ones that show red axil pigmentation, although their seeds have been collected on a green-axil seed parent (Figure 1d). We expect that these plants are hybrid plants, since they show the dominant phenotype they have inherited from the pollinator. Additionally, we considered at least one green-axil plant from each cross as control for genotyping. As a second step, we used the publicly available Insertion-Deletion (InDel) markers described in Zhang et al. (2017) to confirm the genotype of the F_1 plants (Table S1). The parental lines of each cross were

first screened with the InDel markers to find the polymorphic markers for each cross combination. These markers were then used for the identification of true F_1 plants from the putative ones (Figure 3). Every PCR reaction with the InDel markers had a total volume of 20 μl with the following amplification conditions: 94°C for 5 min as initial denaturation, 35 cycles of: 30 s at 94°C, 30 s at primer pair annealing temperature and 30 s at 72°C and a final extension step of 10 min at 72°C.

2.5 | Success rate evaluation and statistical analysis

For the calculation of success rates and selection accuracy and for their statistical analysis, we used the statistical software R (Team, R. C, 2019). We used appropriate generalized linear models (McCullagh & Nelder, 1989) with the parental accessions combination and crossing method as influence factors. No interaction effects were assumed. The residuals were assumed to follow a binomial distribution. For success rates, the distribution is based on the number of confirmed F_1 plants and the total number of the plants phenotyped for each cross. For selection accuracy, the distribution is based on the number of confirmed F_1 plants using molecular markers and the total number of putative F_1 plants for a given cross, which were selected using the phenotypic selection method. In this context and in order to enhance the model estimates, we used a Bayesian generalized linear model described by Gelman et al. (2008) for data analysis. Based on the model for the success rates, an analysis of variances was conducted, followed by multiple contrast tests (Bretz et al., 2011) in order to (a) identify the superior parental combinations and (b) compare the crossing methods.
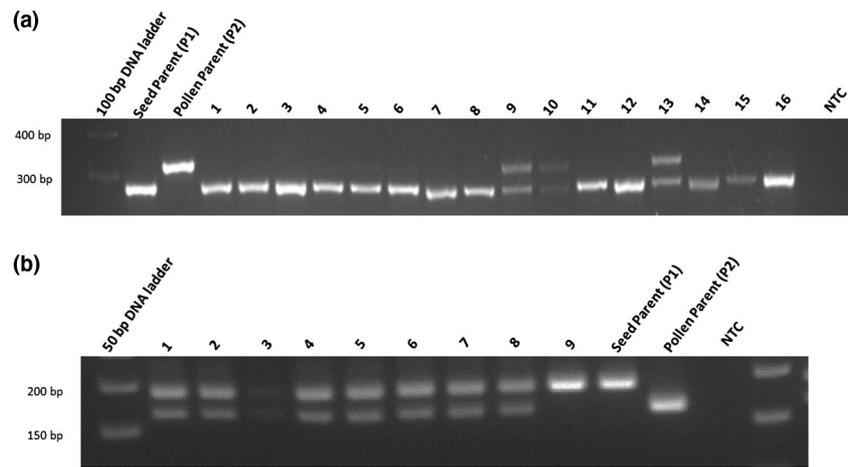
FIGURE 3 Exemplary gel pictures of the result of the selection of F_1 plants based on InDel markers. (a) Seed parent: 171,115, pollen parent: 170,985, lanes 1 to 15: putative F_1 plants, lane 16: selfed control plant, lanes 9, 10, 13: true F_1 plants. Primer combination: JAAS4, Expected amplicon size: 280 and 323 bp. 3% agarose gel was run for 60 min at 100 V. NTC: non-target control. (b) Seed parent: 171,010, pollen parent: 170,916, lanes 1 to 8: putative F_1 plants, lane 9: selfed control plant, lanes 1, 2, 3 (after repeating the PCR for a more clearer result), 4, 5, 6, 7, 8: true F_1 plants. Primer combination: JAAS5, Expected amplicon size: 189 and 164 bp. 3% agarose gel was run for 60 min at 100 V. NTC, non-target control

2.6 | Construction of phylogenetic tree

Twenty-out of 23-investigated accessions in this study were previously sequenced using short reads (paired-end, 2×150 bp) Illumina NovaSeq technology at a genome coverage of ~8X. The mapping of those reads together with whole genome resequencing of a diversity panel of 894 quinoa accessions against an updated version of the coastal quinoa reference genome (Jarvis et al., 2017) was used to identify SNP variants using an automated pipeline compiled from Genome Analysis Toolkit (GATK - v4.0.1.1) suite (McKenna et al., 2010), described and available on GitHub (https://github.com/IBEXCluster/IBEX-SNPcaller/blob/master/workflow.sh). To obtain high confidence variants, SNPs with no more than 10% missingness at the genotype level and biallelic alleles only were considered. The twenty accessions investigated in this paper were then extracted, and further filtering of variants for minor allele frequency (MAF) less than 5% was applied using vcftools (v0.1.17; Danecek et al., 2011), resulting in a total of 1,602,914 high-quality SNPs for phylogenetic tree construction. First, we used snpgdsIBS function in the R package SNPrelate (Zheng et al., 2012) to calculate a pairwise dissimilarity matrix. Then we performed clustering using snpgdsHCluster function, and the phylogenetic tree was created with snpgdsCutTree function with the following criteria; z.threshold = 15, outlier.n = 5, n.perm = 5,000.

3 | RESULTS

We produced in total 214 F_1 hybrids from 30 crosses between 23 parents using three crossing methods. All the hybrid plants were bag-isolated and selfed to produce 30 F_2 populations. The crossing parents were selected based on their axil pigmentation which had been determined before (Table 1). We considered three crossing methods (no emasculation, hand emasculation and warm water emasculation) and the parental combinations as sources of variation. Our results showed that both parental combinations and crossing methods have a significant effect on the success rate of the crossing experiment (ρ = <2.20E-16 and 2.32E-10, respectively). We used a two-step method for the identification of F_1 plants from all the crosses. As axil pigmentation has been reported to have a dominant monogenic inheritance (Simmonds, 1971), we expect all the F_1 plants from a cross between a green-axil seed parent and a red-axil pollinator to have red axils (Figure 1d). Therefore, we only considered genotyping with the molecular markers for the red-axil plants together with a green-axil plant as control. For genotyping, we used six publicly available InDel markers (Zhang et al., 2017), which were polymorphic between the parents (Table 2 and Figure 3). Markers produced PCR product sizes of 168 to 323 bp with an insertion-deletion size of 25 to 100 bp. Only plants heterozygous for each marker were regarded as true hybrids. In total, we performed genotyping of 302 putative F_1 plants using the InDel markers, out of which 214 showed the expected genotype of an F_1 plant and therefore, were called as true F_1s. Hence, we calculated the efficiency rate of 70.86% for axil pigmentation for selection of F_1 plants, by dividing the number of true F_1 plants by the total number of putative F_1 plants for each cross.

We also examined the suitability of hypocotyl colour for detecting hybrid plants (Supplementary Figure 1). Here we considered eight different crosses between seed parents with green (recessive) hypocotyl and pollinators with red (dominant) hypocotyl colours (Table S2). We used the no emasculation method for performing these crosses. The putative F_1 plants were first selected based on their red hypocotyl colour and later confirmed by molecular markers. We recorded a selection accuracy rate of 88.89%, which is

TABLE 2 Success rate and accuracy rate of axil pigmentation for different crossing combinations

Seed parent	Pollinator	Polymorphic marker	Success rate of no emasculation (%)	Success rate of water emasculation (%)	Success rate of hand emasculation (%)	Selection accuracy rate of axil pigmentation (%)	Number of F_1 hybrids
170867	171519	JAAS4 (280, 323)	0.76	2.27	5.31	48.19	4
170886	170876	JAAS7 (191, 291)	0.14	0.42	1.02	89.24	1
170886	170916	JAAS7 (191, 291)	6.06	16.31	31.99	99.74	122
170886	171008	JAAS7 (191, 291)	0.13	0.40	0.97	98.04	2
170886	180450	JAAS7 (191, 291)	0.02	0.06	0.14	97.58	0
171010	170875	JAAS1 (168, 256)	0.38	1.14	2.71	98.28	4
171010	170916	JAAS5 (189, 164)	0.78	2.33	5.44	98.59	8
171010	170985	JAAS1 (168, 256)	0.09	0.26	0.63	16.15	0
171024	170875	JAAS5 (189, 164)	0.45	1.34	3.18	46.02	1
171024	171008	JAAS4 (280,323)	14.83	34.48	55.94	84.93	2
171024	171198	JAAS4 (280, 323)	8.64	22.22	40.80	78.21	2
171079	170876	JAAS1 (168, 256)	1.24	3.66	8.41	49.36	1
171079	171139	JAAS7 (191, 291)	0.11	0.33	0.80	97.58	0
171079	171599	JAAS18 (252, 209)	0.29	0.87	2.08	89.24	1
171115	170876	JAAS4 (280,323)	0.56	1.68	3.97	57.62	2
171115	170985	JAAS4 (280, 323)	0.39	1.16	2.76	25.91	3
171115	171139	JAAS7 (191, 291)	0.20	0.60	1.45	6.71	0
171115	171205	JAAS5 (189, 164)	0.65	1.94	4.57	93.16	10
171115	171599	JAAS4 (280, 323)	0.07	0.22	0.53	1.60	1
171169	170876	JAAS1 (256, 168)	0.02	0.05	0.12	97.58	0
171169	170916	JAAS7 (291, 191)	1.36	4.00	9.14	34.14	13
171214	170875	JAAS28 (280, 260)	0.17	0.53	1.26	97.85	1
171214	171008	JAAS7 (191, 291)	0.15	0.46	1.09	97.58	0
171214	180322	JAAS7 (191, 291)	0.22	0.65	1.56	97.58	0
171230	170876	JAAS4 (280, 323)	1.28	3.77	8.63	90.43	7
171230	171599	JAAS5 (189, 164)	2.39	6.89	15.16	48.22	12
171510	171008	JAAS4 (280, 323)	0.05	0.15	0.35	85.88	0
171605	170875	JAAS5 (189, 164)	0.09	0.27	0.65	97.58	0
171605	170916	JAAS1 (168, 256)	0.82	2.44	5.70	95.84	15
171605	171008	JAAS5 (189, 164)	0.22	0.67	1.60	98.04	2

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TABLE 3 Statistical analysis of success rate to compare different crossing methods

Comparisons	Logit estimate	Standard error	ρ-value
Hand emasculation versus no emasculation	1.987	0.361	<.0001***
Water emasculation versus no emasculation	1.106	0.391	.0118*
Water emasculation versus hand emasculation	−0.881	0.195	<.0001***

*, ***significant at α = 0.05 and α = 0.001 respectively. The ρ-values are based on appropriate multiple contrast tests (Bretz et al., 2011).

comparable to the accuracy rate we reported for axil pigmentation, considering the low number of investigated crosses for hypocotyl colour.

The comparison of different crossing methods revealed significant differences between all three methods (Table 3). Hand emasculation was the most successful crossing methods followed by warm water emasculation. The success rate of the hand emasculation method ranged from 0.12% to 55.94%, while crosses that were performed using warm water emasculation showed a success rate between 0.05% to 34.48%. No emasculation method turned out to be not suitable because of the low success rate of 0.02 to 14.83% (Table 2).

The efficiency of hybrid seed production was largely varying between cross combinations. The cross 171,024 × 171,008 was the most successful crossing combination with the average success rate of 35.08% over all three crossing methods, while 171,169 × 170,876 produced the least number of F_1 plants with an average success rate of 0.06% (Table 2). To select the most successful parental combinations for the production of hybrids, we identified the crosses, which showed a higher success rate over all three crossing methods compared to the overall mean of the whole experiment. Five crossing combinations produced hybrid seeds with a significantly higher success rate compared to the mean success rate of the experiment (Table 4).

We wanted to know if the phylogenetic relationship of the parents plays a role in the success rate of their cross. Therefore, we used 1.6 million SNPs derived from the whole genome re-sequencing of 20 out of the 23 investigated accessions in this study for phylogenetic analysis. The individual's dissimilarity and coancestry coefficient grouped the accessions in two main clusters (group I and group II; Figure 4). Nine out of the ten (90%) accessions in group I were originated from Peru or Bolivia, while all but one of the Chilean accessions investigated in this study were grouped in group II. Group I represents quinoa highland accessions in our experiment, while accessions in group II belong to the coastal quinoa population (Jarvis et al., 2017). We found crosses between more related accessions (e.g. 170,886 × 170,916, mean success rate of 18.12%) to produce

TABLE 4 Success rates of individual crossing combinations in comparison to the overall success rate of the crossing experiment

Seed parent	Pollinator	Logit estimate	Standard error	ρ-value
170867	171519	0.603	0.382	.850
170886	170876	−1.093	0.787	1.000
170886	170916	2.730	0.219	<.001***
170886	171008	−1.142	0.640	1.000
170886	180450	−3.112	1.516	1.000
171010	170875	−0.097	0.503	1.000
171010	170916	0.629	0.397	.844
171010	170985	−1.569	1.417	1.000
171024	170875	0.069	0.835	1.000
171024	171008	3.724	0.852	<.001***
171024	171198	3.113	0.942	.014*
171079	170876	1.097	0.959	.992
171079	171139	−1.334	1.416	1.000
171079	171599	−0.366	0.800	1.000
171115	170876	0.299	0.674	1.000
171115	170985	−0.079	0.573	1.000
171115	171139	−0.737	1.470	1.000
171115	171205	0.445	0.365	.983
171115	171599	−1.756	0.788	1.000
171169	170876	−3.226	1.528	1.000
171169	170916	1.188	0.324	.004**
171214	170875	−0.877	0.810	1.000
171214	171008	−1.021	1.432	1.000
171214	180322	−0.658	1.479	1.000
171230	170876	1.126	0.409	.085
171230	171599	1.762	0.335	<.001***
171510	171008	−2.157	1.397	1.000
171605	170875	−1.544	1.415	1.000
171605	170916	0.678	0.312	.366
171605	171008	−0.637	0.643	1.000

Note: For the statistical analysis, the logit estimates of the success rates were calculated. *, **, ***: parental genotype combinations that show a higher success rate compared to the mean of all the crosses at α = 0.1, α = 0.05, α = 0.01 and α = 0.001, respectively. The ρ-values are based on appropriate multiple contrast tests (Bretz et al., 2011).

similar success rate to the crosses between further distant accessions (e.g. 171,024 × 171,198, mean success rate of 23.88%).

4 | DISCUSSION

For cultivation of quinoa in different environmental conditions, careful design of region-specific breeding programmes is required. Since all line breeding programmes start with crosses to create genetic variation and combine desired characters from different accessions,
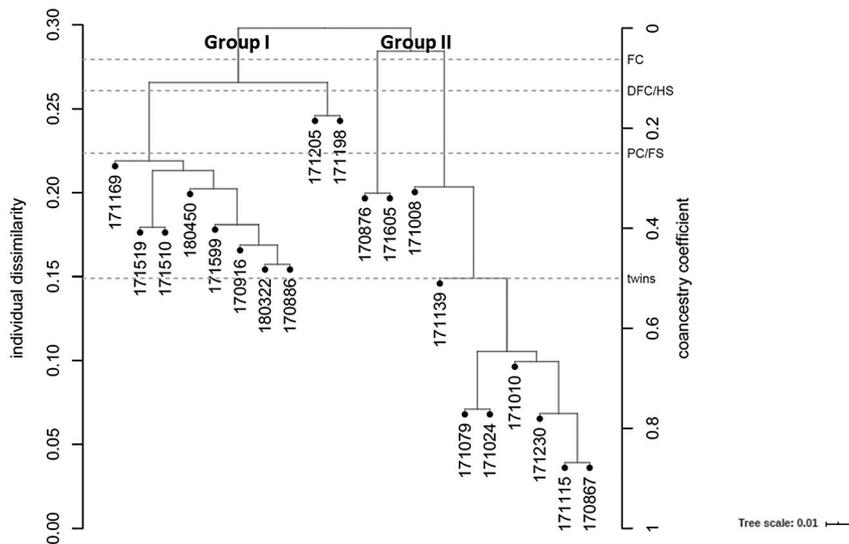
FIGURE 4 Phylogenetic relationship of the crossing parents. DFC, double first cousin; FC, first cousin; FS, full siblings; HS, half siblings; PC, parent-child

it is crucial to develop a simple method for crossing and hybrid seed detection. Manual emasculation has been suggested as a promising method for production of hybrid seeds. However, hand emasculation is reported to be very difficult in quinoa due to its compact inflorescence and its tiny florets (Jacobsen & Stølen, 1993), while warm water emasculation has been considered ineffective due to the damage to the inflorescence (Fleming & Galwey, 1995). Despite of the challenges of the hand emasculation of quinoa flowers, attempts have been made to develop F_2 populations after hand crossing in quinoa. A detailed instruction for hand emasculation of quinoa flowers under controlled conditions is available (Peterson et al., 2015). Nonetheless, this protocol only describes the crossing procedure and does not report on further selection of F_1 plants using molecular markers. Here, we present a two-step selection strategy for the selection of true F_1 plants based on morphological and molecular markers. In this way, we created 30 segregating populations from crosses between closely related as well as distantly related quinoa accessions.

We reasoned that two components (parental combination and crossing method) would account for the crossing success. Quinoa is an autogamous species with highly variable outcrossing rates (0.5 to 17%) across different accessions (Murphy et al., 2016). In a crossing experiment, parental accessions with a high outcrossing rate can be used to increase the possibility of cross pollination. In general, the rate of outcrossing can be influenced by (a) inflorescence type and proportions of different flower types; (b) percentage of hermaphrodite flowers; (c) the amount of androsterile hermaphrodite flowers; (d) presence of cleistogamy and self-incompatibility; and (e) the environmental conditions such as temperature (Gomez-Pando, 2015). Quinoa accessions, which produce less cleistogamous and more chasmogamous flowers with exposed anthers and abundant pollen over a longer period of time, would be ideal candidates as pollinators in crossing programmes. Additionally, accessions with higher number of pistillate flowers would be more suitable as seed parents in crossing programmes. We would suggest phenotyping these traits in different quinoa accessions to identify putative candidates as crossing parents for breeding programmes in quinoa.

Apart from mating type and floral morphology, pollinators with dominant qualitative morphological traits can facilitate the identification of F_1 plants. In the current experiment, we selected different parents from highland and coastal origin displaying high genetic diversity (Jarvis et al., 2017). To reduce the molecular marker screening workload, we considered axil pigmentation for the first selection step of the F_1 plants. It is important to mention, that the identification of red axil plants is not always easy. In some cases, axils of putative F_1 plants display a pink colour, which makes it hard to distinguish them from the green axils of selfing progenies. Moreover axil pigmentation may appear sporadically, which makes it difficult to detect (Peterson et al., 2015). Therefore, the selection accuracy due to axil pigmentation was highly variable and not suitable as a sole criterium to detect hybrids. There are other morphological traits like stem colour, inflorescence colour, saponin content or seed colour, which can be considered for the selection of F_1 plants. However, for the efficient identification of F_1 plants, the morphological trait used for selection should be easy to phenotype. This does not apply for traits, which can only be phenotyped late in the development of the plant (e.g. inflorescence colour, saponin content and seed colour). Moreover traits like stem colour and inflorescence colour can be influenced by the environment, which reduces their usefulness as selectable markers. We also considered hypocotyl colour for the first stage of selection of F_1 plants in a few numbers of crosses and recorded a comparable selection accuracy rate to the one reported for axil pigmentation. Therefore, both axil pigmentation and hypocotyl colour are efficient for the initial selection of F_1 plants. However, while hypocotyl colour can be phenotyped much earlier compared to axil pigmentation (already one week after sowing), the phenotyping for this trait might be very challenging since it is not easy to discriminate between green and red hypocotyls in very young seedlings.

We have used molecular markers in a second step to confirm the hybrid nature of the plants. Identification of F_1 plants using the molecular markers can be performed faster in comparison to selection based on some morphological markers, where one can first see the traits in later plant's developmental stages (e.g., seed colour and

inflorescence colour). The most efficient molecular markers are the PCR-based markers like Insertion-Deletion (InDel), Cleaved Amplified Polymorphic Sequence (CAPS) or Kompetitive Allele Specific PCR (KASP) markers that can screen the F_1 plants in a cheap and timely manner. Selection of F_1 plants by InDel markers is a quick, cheap, and reliable technique, because PCR fragments can be simply visualized on an agarose gel. Since we applied bag isolation and therefore excluded any unintended cross-pollination, we could limit the analysis to only polymorphic InDel marker. Stetter et al. (2016) considered KASP for confirmation of hybrid plants in grain amaranth. In our hands, InDel markers offer a time- and cost-effective approach compared to KASP markers. However, if bag isolation is not possible due to the experimental conditions, more marker loci should be considered to exclude hybrid seeds produced through pollination by unknown pollinators. Using a two-stage selection strategy for hybrid plants considering a morphological trait as the first selection step would reduce the costs of genotyping. Nevertheless, this would limit the number of crosses that can be performed, since the pollinator and seed parent should always show contrasting phenotypes for the morphological trait. In the future, we suggest relying solely on molecular markers for selection of hybrid plants in crossing experiments. To speed up the screening procedure and to reduce the costs, we suggest considering pooled leaf samples from several putative F_1 plants for DNA isolation and genotyping. In case of positive genotyping results for a pool, all the single plants in that pool should be individually genotyped again to identify the true F_1 plants.

In our study, the parental combination had a significant effect on the success rate of the cross. We identified five parental combinations, which produced significantly higher numbers of hybrid plants. By comparing the most successful crosses, it is evident that the accessions 171,024 and 170,916 were present in more than one significantly successful cross as seed parent and pollinator, respectively. Among the five most successful crosses, two crosses were made between highland ecotypes, while one cross was made between the coastal accessions and two crosses were made between different ecotype groups. Therefore, considering the material explored in the current study, we did not see any associations between the phylogenetic relationship of the accessions and the success rate of the crosses. This suggests that crossing success in quinoa will be independent of the parental origin. It is noteworthy that hand emasculation has been successfully used to generate interspecific hybrids between *C. quinoa* and *C. berlandieri*, which opens new perspectives for breeding quinoa for disease resistances (Bonifacio, 2003).

Hand emasculation was the most successful crossing method in the current study, followed by warm water emasculation and no emasculation, which was in line with the previous report in grain amaranth (Stetter et al., 2016). However, we recorded a lower success rate for our most efficient method (maximum success rate of 55.94% for hand emasculation) compared to the reported success rates in amaranth (74% for hand emasculation; Stetter et al., 2016). We believe, this difference is due to slight differences in the execution of the crossing methods. In the previous study in amaranth, the emasculation was repeated after seven days of the first emasculation and any flowers

developed after the emasculation were removed. In our study due to technical reasons and the high number of crosses that we performed, repeated emasculation of the seed parents and removal of extra flowers were not technically feasible. Therefore, we expected a higher percentage of selfing seeds in the crossing progenies, which was a reason to consider 280 seeds per crossing event for identification of F_1 plants. In future experiments, we will consider multiple emasculation events and elimination of the intact flowers on the seed parents, to increase the success rate of the crosses. Nevertheless, it is evident that the hand emasculation significantly increases the success rate of the crosses and should be considered to assure the production of hybrid seeds.

Differences in flowering time and height between the crossing parents can complicate the crossing procedure. In our experiment, we considered earlier sowing of the late-flowering parents and five sowing dates in intervals of seven days for early flowering parents to synchronize the flowering time between the parental combinations. Considering the height, we always placed the parental combinations in a way that the pollinator's flowers are above the seed parent's flower or at the same level, using tables and boxes. We performed the current experiment under semi-controlled environmental conditions in a greenhouse during summer. The long day conditions during summer promote later flowering and continuous vegetative growth of quinoa, which is originally a short-day plant. Therefore, we recommend growing the plants under short-day conditions. In this way, one can accelerate the growth cycle of quinoa plants and restrict the vegetative growth, which results in reduced height of the crossing parents. Additionally, exposure to shorter hours of light would reduce the number of flower clusters and accelerate the flowering and seed set of the plants, which is in general desired for the crossing experiments. Moreover sowing the seeds of the crossing parents in smaller pots also results in earlier flowering. Stetter et al. (2016) could significantly shorten the growth cycle of amaranth plants using the same strategy. A speed breeding protocol for shortening the growth cycle of quinoa is already available (Ghosh et al., 2018). However, this protocol is only designed for day-neutral/long-day quinoa accessions. Using the measures mentioned above, we expect to be able to shorten the growth cycle of short-day quinoa accessions, which encompass the majority of quinoa germplasm.

We produced 30 segregating populations from crosses between genetically diverse quinoa accessions, which can serve as starting material for breeding programmes in quinoa. The improved progenies of the crosses can be selected and investigated in consecutive generations using the established breeding methods like pedigree or single seed descent. Moreover the F_2 populations and their progenies can be used for QTL mapping and identification of candidate genes for agronomically important traits in quinoa.

CONFLICT OF INTEREST

The authors declare that there is no competing interest.

AUTHOR CONTRIBUTIONS

NE designed the experiment, performed the crosses and wrote the manuscript. MH performed the statistical analysis. DS calculated the phylogenetic relationship between the parental accessions. NM performed genotyping of the progenies using the molecular markers. ER produced the SNP file for the parental accessions. CJ supervised the study and critically revised the manuscript. All authors read and approved the manuscript.

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