

1 **Inference of the distribution of fitness effects of mutations is affected by SNP filtering methods,**
2 **sample size and population structure**

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18 [Total word counts for the main body of the text: 7367 \(excluding references\)](#)

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33 **Abstract**

34 The distribution of fitness effects (DFE) of new mutations has been of interest to evolutionary
35 biologists since the concept of mutations arose. Modern population genomic data enable us to
36 quantify the DFE empirically, but few studies have examined how data processing, sample size
37 ~~and~~ cryptic population structure might affect the accuracy of DFE ~~inference~~. ~~We used simulated~~
38 ~~and empirical data (from Arabidopsis lyrata) to show the effects of missing data filtering,~~
39 ~~sample size, number of SNPs and population structure on the accuracy and variance of DFE~~
40 ~~estimates. Our analyses focus on three filtering methods~~ – downsampling, imputation and
41 subsampling – ~~with~~ sample sizes ~~of~~ 4 ~ 100 individuals. We show that (1) ~~the choice of missing-~~
42 ~~data treatment directly affects the estimated DFE, with downsampling performing better than~~
43 ~~imputation and subsampling;~~ (2) the estimated DFE ~~is~~ less reliable in small samples (<8
44 individuals), and ~~becomes~~ unpredictable ~~with~~ too few SNPs (<5000); ~~and~~ (3) population
45 structure ~~may~~ skew the ~~inferred~~ DFE toward more strongly deleterious mutations. We ~~suggest~~
46 that future studies ~~should consider downsampling for small datasets, and use samples larger~~
47 ~~than 4 (ideally larger than 8) individuals, with more than 5000 SNPs~~ in order to improve the
48 robustness of ~~DFE inference~~ and enable comparative analyses.

49
50 **Key words:** DFE, missing-data treatment, population structure, sample size, ~~SLiM simulation~~.

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1 INTRODUCTION

The distribution of fitness effects (DFE) of new mutations can be described as the probability that a new mutation will have a specific effect on the fitness of an individual. This probability distribution affects the accumulation of genetic variation, and can thus directly impact the evolutionary trajectory of organisms (Bataillon & Bailey, 2014; Keightley & Eyre-Walker, 2007; Ohta, 1992). Understanding the DFE is integral to understanding molecular evolution and remains an important focus in modern evolutionary theory (Chen et al., 2020; Halligan & Keightley, 2009; Kimura, 1968; Ohta, 1973). To date, the arguably most popular methods of inferring the DFE are based on contrasting frequencies of putatively neutral and selected polymorphisms presented as a site frequency spectrum (SFS), describing how commonly mutations of different frequencies occur in a population (Gutenkunst et al., 2009; Keightley & Eyre-Walker, 2007; Kim et al., 2017; Tataru & Bataillon, 2019). Since the SFS can be affected by both neutral and selective processes, most methods use the SFS of synonymous mutations to estimate a demographic model representing the effects of population size changes and genetic drift. Meanwhile, the SFS of non-synonymous mutations are assumed to be shaped by both neutral and selective processes, and can therefore be used to estimate the DFE of non-neutral mutations after demography and drift have been accounted for (Boyko et al., 2008; Huang et al., 2021; Keightley & Eyre-Walker, 2007; Kim et al., 2017; Schneider et al., 2011; Tataru & Bataillon, 2019). However, factors other than demography and selection may also affect the shape of the SFS and thus the estimated DFE.

First, SFS-based DFE inferences require that datasets contain no missing sites – all individuals must have complete data for all loci that are to be analysed. Since sequencing techniques are imperfect, such datasets are uncommon (probably non-existent) in empirical population genomics. As a result, missing-data treatment is an essential first step of data processing. To obtain a complete dataset, the data are treated either by filtering out some portion of the data (sub- or downsampling), or filling in the “gaps” using an algorithm such as imputation, see section 2.2 Missing-data treatment methods). Depending on how the treatment is performed, there is a risk of altering the relative allele frequencies in the dataset, yielding misleading results (Johri et al., 2021; Larson et al., 2021). Recent studies on DFE have applied different data processing methods; for example see Hämälä & Tiffin (2020) for imputation, and Gossmann et al. (2010) for downsampling. However, it is unknown whether and how the different methods influence DFE estimates.

Second, the sizes of datasets used in published DFE studies vary enormously, from as few as two to several hundred individuals (Chen et al., 2017; Hämälä & Tiffin, 2020). The SFS

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251 is highly sensitive to sample size, but the minimum number required to achieve stable DFE
 252 estimates remains undetermined (but see Kutschera et al. 2020). Similarly, the number of
 253 polymorphic sites necessary for reliable DFE estimation is largely unknown. While some
 254 studies of model species use whole genome sequencing with millions of single nucleotide
 255 polymorphisms (SNPs) available for analysis (Hämälä & Tiffin, 2020), others may only include
 256 a few hundred SNPs (Eyre-Walker & Keightley, 2009; Gossmann et al., 2010). Therefore,
 257 investigating the impact of sample size (both the number of individuals and sites/SNPs) on DFE
 258 estimates is crucial for reliable and accurate DFE estimation.

259 Finally, most methods of SFS-based DFE estimation first estimate a Wright-Fisher
 260 demographic model from the neutral variation in order to control for neutral factors affecting
 261 the SFS (Keightley & Eyre-Walker, 2007; Tataru & Bataillon, 2019). Such models assume that
 262 mating occurs at random in panmictic populations, even though complete absence of population
 263 structure is likely rare in wild samples. For example, sampling from a large area is preferred for
 264 drawing general conclusions about population genetic dynamics, but it increases the likelihood
 265 of including genetic structure in the sample (Perez et al., 2018; Zhao et al., 2020). If cryptic
 266 genetic clusters are unwittingly included, the demographic model estimated from the data
 267 would not fulfil the assumptions underlying the Wright-Fisher model, and subsequent DFE
 268 estimates might be biased. However, population stratification has not to our knowledge been
 269 examined as a potential factor affecting the accuracy of DFE inference.

270 In this study we test whether and how data processing methods, sample size, SNP
 271 number and population structure influence the results of DFE inference, to raise awareness of
 272 their potential confounding effects. We used whole genome re-sequencing data from two
 273 populations of *Arabidopsis lyrata* (subsp. *petraea*) to create multiple datasets (Fig. 1) with (1)
 274 three different methods of missing-data treatment – downsampling, imputation and
 275 subsampling – under different filtering thresholds; (2) different numbers of randomly sampled
 276 individuals and sites; and (3) samples with induced population stratification, to be contrasted
 277 with uniform, single populations. Then, we conducted forward simulation in SLiM 4.0 (Haller
 278 & Messer, 2023) to create a population with a known DFE that matches DFEs estimated in *A.*
 279 *lyrata*. Using this known DFE, we evaluate the accuracy of DFE estimates resulting from the
 280 different data manipulations. By contrasting the results obtained from the different procedures,
 281 we aim to answer the following questions: (1) Do data processing methods and missing-data
 282 filtering thresholds affect DFE estimation, and if so, how? (2) How many individuals and SNPs
 283 are needed to reach an accurate DFE estimate? and (3) Does population structure affect the
 284 DFE, and if so, how? Our results illustrate the importance of careful consideration of all steps

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Deleted: , yet there are no general evaluations of the minimum sample size necessary for achieving stable DFE estimates (but see Kutschera et al. (2020)). Similarly, the effect of the numbers of sites and SNPs on DFE estimation is largely unknown. Studies of model species frequently use whole genome sequencing with millions of SNPs available for analysis (Hämälä & Tiffin, 2020) while other studies may include only a few hundred SNPs (Eyre-Walker & Keightley, 2009). Examining the potential confounding effects of sample size (i.e. both the numbers of individuals and sites) on DFE estimates is therefore of great value.¶

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318 in genomic data processing and analysis, both when performing [DFE inference](#) and [when](#)
319 interpreting [its](#) results.

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321 [2 MATERIALS AND METHODS](#)

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322 [2.1 Genomic dataset and basic quality control](#)

323 We downloaded the whole genome re-sequencing data for two populations of the perennial,
324 diploid, obligately outbreeding *Arabidopsis lyrata* subsp. *petraea*, 29 individuals from Austria
325 and 16 individuals from Norway, from the NCBI SRA database (Table S1). The quality of the
326 sequence reads was first assessed with FastQC
327 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Adapter sequences and low-
328 quality bases were removed using fastp v0.23.0 ([Chen et al., 2018](#)) with the parameters “-q 20
329 -l 36 --cut_front --cut_tail -c”. Clean reads were mapped to the *A. lyrata* v.1.0 genome
330 (<https://plants.ensembl.org/>) using the BWA-MEM algorithm with default parameters (Li,
331 2013). PCR duplicates were removed using Picard MarkDuplicates (<http://broadinstitute.github.io/picard/>). Reads around putative insertions and deletions were locally realigned using
332 RealignerTargetCreator and IndelRealigner in the Genome Analysis Toolkit (GATK v.3.7-0;
333 [Van der Auwera et al., 2013](#)). Variants were called using the SAMtools and BCFtools pipeline
334 as described in (Li, 2011). Several filtering steps were performed to minimize genotyping
335 errors: indels and SNPs with mapping quality (MQ) <30 were removed, genotypes with
336 genotype quality (GQ) <20 or read depth (DP) <5 were masked as missing, and all SNPs with
337 a missing rate above 50% or allele number above 2 were removed. After these basic filtering
338 steps, a total of 122,432,856 sites ([including invariant sites](#)) were retained in the 45 samples for
339 the following analyses.

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342 [2.2 Missing-data treatment methods](#)

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343 Missing genotypes are common in genomic datasets and should be eliminated before generating
344 an SFS. We tested three methods to treat missing values [on the same original datasets](#) –
345 *downsampling*, *imputation* and *subsampling* (Fig. 1a, Fig. 2), and then compared the DFE
346 inferred from each resulting dataset using bootstrapped 95% confidence intervals (CIs).

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Deleted: . Since all filtering was performed on the same original datasets for each population, any significant differences in the estimated DFE could be assumed to result solely from the filtering procedure.

347 *Downsampling* is performed by randomly selecting n genotypes at each site without
348 replacement (Keightley & Eyre-Walker, 2007); sites with fewer than n genotypes available are
349 removed. A 75% downsampling threshold in a sample size of 100 individuals means that 75
350 random genotypes are sampled at each site (Fig. 2). Sites that contain < 75 genotypes are
351 removed. In this study, we applied downsampling at thresholds 75%, 66%, and 50% on both

366 Austrian and Norwegian datasets. The same set of sites were kept and analyzed in both
367 populations, making direct comparisons of the DFE between populations possible.
368 Downsampling was performed using a Python script available on Dryad (Papadopoulou &
369 Knowles, 2015) with minor modification (https://github.com/hui-liu/Bioinformatics-Scripts/blob/master/Scripts/Python/sampleDownMSFS_Hui_final.py).

371 *Imputation* refers to the statistical inference (“filling in”) of missing genotypes using
372 the available linkage information from successfully genotyped samples (Fig. 2). We tested
373 threshold 70%, 80% and 90% on the *A. lyrata* datasets (i.e. excluding sites with less than 70%,
374 80% and 90% genotype information available), and filled in the missing genotypes at all other
375 sites using Beagle v5.1 (Browning et al., 2018) with default parameters. We performed
376 imputation using all individuals from both populations, as imputation accuracy tends to increase
377 with sample size, as shown by previous studies (Pook et al., 2020).

378 *Subsampling* works in two steps: 1) Individuals that are missing more than a prescribed
379 fraction of their genotype information are excluded, and 2) for the individuals remaining, any
380 site with a missing genotype is removed (Fig. 2). This means that the size of a subsampled
381 dataset is highly dependent on the individual missing rates and the distribution of missing data
382 across the genome. We first calculated the missingness on a per-individual basis using the
383 parameter “--missing-indv” in VCFtools (Danecek et al., 2011). We then extracted the
384 individuals that had missing rates below the threshold value using “--keep”, and finally, we
385 removed all sites containing missing genotypes by setting the parameter “--max-missing 1” in
386 VCFtools. In the *A. lyrata* dataset, we tested four maximum missing rates per individual – 10%,
387 15%, 20% and 25% (Note: no individual had more than 25% missing data). Note that with
388 higher subsampling thresholds, more individuals but fewer sites are retained (Fig. 1a).

390 2.3 Sample size and SNPs number

391 To decouple the potential effects of the number of individuals and/or sites on DFE estimation,
392 we randomly sampled 4, 8, 12, 16, 20, 24 or 29 (all) individuals and/or 1K, 10K, 100K, 1M,
393 10M or 55.0M sites from the Austrian population subsampled at a maximum missing rate of
394 25% per individual (Fig. 1). To investigate the effect of sample size, we kept all sites and
395 compared samples with different numbers of individuals (4 - 29). Conversely, to investigate the
396 effect of the number of SNPs, all 29 individuals were kept and a randomly chosen subset of 1K
397 to 10M sites were extracted. Finally, the same subsets of 1K to 10M sites were extracted from
398 a dataset with only 4 individuals. By comparing the DFEs from 4 vs. 29 individuals for each

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Deleted: As the accuracy of the imputation could be affected by the missing rate of sites, we filtered the VCF file with three different missing rates, allowing a maximum of 10%, 20% and 30% missing for each site, before imputation. These missing rates correspond to our thresholds of 90%, 80% and 70% data required per site. Imputation was performed with Beagle v5.1 (Browning et al., 2018) using default parameters. To keep the same set of sites in both populations, imputation was performed on combined dataset of both populations.

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Deleted: and 55.0M sites with no missing values. We then generated 19 sets of VCFs for several combinations of individuals and sites (Fig. 1b), including:¶
Seven VCFs each containing 4, 8, 12, 16, 20, 24 or 29 (all) individuals, randomly chosen from the initial population. We then analysed the DFE for each of the datasets

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Six VCFs containing

Deleted: either 1K, 10K, 100K, 1M, 10M or 55.0M sites with no missing data, respectively. With these, we compared DFE estimates of the same set of individuals but across different numbers of sites.¶
Six VCFs, using the same type of analysis as in B, with

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439 set of sites, we could see the combined effects of the number of individuals and sites on the
440 estimated DFE and confidence intervals (Fig. 3).

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442 **2.4 Manipulating population structure**

443 To gain an overview of the genetic differentiation between the Austrian and Norwegian
444 populations, we performed a principal component analysis (PCA) on the 45 sampled individuals
445 using Eigensoft v.6.1.4 (Price et al., 2006). The dataset was filtered at a maximum missing rate
446 of 20% per site and a minor allele frequency (MAF) ≥ 0.05 , retaining 3,921,575 SNPs for the
447 PCA. To investigate whether population structure affects DFE estimates, we randomly selected
448 three different subsets (labelled a, b and c) of 10 and 15 individuals from each of the Austrian
449 and Norwegian populations, imputed at an 80% threshold. Single sets from each population
450 were then combined to form 12 new merged populations with four different configurations (Fig.
451 1c): 10 Austrian + 10 Norwegian individuals, 10 Austrian + 15 Norwegian individuals, 15
452 Austrian + 10 Norwegian individuals, and 15 Austrian + 15 Norwegian individuals, each with
453 three replicates. We then estimated the DFE for each subset as well as all merged samples.

Deleted: to form 12 subsets in total: three sets of 10 individuals from Austria (Aus10 a, b and c), three sets of 15 individuals from Austria (Aus15 a, b and c), and the same for the Norwegian population.

454 Using the single and merged datasets we investigated 1) the effect of sample choice
455 within a geographic population on DFE, by comparing the three replicate subsets from a single
456 population (e.g. replicates *a* vs. *b* vs. *c* of subset Aus10), 2) the effect of each geographic
457 population on the merged population, by comparing the DFE of the merged population to each
458 of the contributing populations (e.g. replicate *c* of merged population Aus10+Nor15 vs.
459 replicate *c* of subsets Aus10 and Nor15), and 3) the effect of population differentiation (F_{ST}) on
460 DFE in the merged population. The weighted F_{ST} between the two contributing subsets in each
461 merged population was calculated using VCFtools.

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463 **2.5 DFE analyses**

464 We used DFE-alpha (Eyre-Walker, & Keightley, 2009), a software that uses a maximum-
465 likelihood approach to determine the shape of the DFE of nonsynonymous mutations. In the
466 simplest model, DFE-alpha assumes that mutations at synonymous sites are selectively neutral
467 and that all non-synonymous mutations are deleterious. DFE-alpha first estimates a simple
468 demographic model using the SFS of neutral mutations to represent the effect of drift. We
469 modelled the effect of recent demographic change on neutral SFS by assuming one step
470 population size change and inferred the fitness of new deleterious mutations at the selected sites
471 from a gamma distribution while simultaneously fitting the estimated parameters for the
472 demographic model. The estimated fitness effects of new mutations are scaled by effective

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485 population size N_e and selection coefficient s as $N_e s$, and divided into four categories:
486 *effectively neutral* ($0 < -N_e s \leq 1$), *slightly deleterious* ($1 < -N_e s \leq 10$), *moderately deleterious*
487 ($10 < -N_e s \leq 100$) and *strongly deleterious* ($-N_e s > 100$). The DFE is presented as the proportion
488 of nonsynonymous mutations that is expected to fall into each of these categories.

489 We generated a folded SFS for a class of putatively neutral reference sites (4-fold
490 degenerate sites) and a class of selected sites (0-fold degenerate sites) for each dataset. We
491 modelled the effects of recent demographic change on the 4-fold sites SFS by assuming a single
492 population size change event and inferred the fitness of new deleterious mutations at the 0-fold
493 sites from a gamma distribution. The 95% CIs for all DFE estimates were calculated by
494 bootstrapping 0-fold and 4-fold sites with replacement for 99 iterations. We performed
495 bootstraps using 999 and 99 iterations in 9 samples and found no discernible difference in CI
496 size; all reported CIs are thus based on 99 iterations.

497

498 **2.6 Simulations in SLiM**

499 To validate the effects of filtering methods and sample size on DFE estimates, we used SLiM
500 4.0 to simulate a population with a known DFE, represented by a gamma distribution with shape
501 (β) and mean (E_s) parameter values matching the DFE estimated in *A. lyrata*. The simulation
502 consisted of a population of 10,000 outcrossing individuals with a genome size of 5 million
503 sites on one contiguous chromosome, and a uniform recombination rate of 4×10^{-8} (Hämälä &
504 Tiffin, 2020). New mutations occurred at a mutation rate of 5.6×10^{-8} and were drawn from a
505 deleterious DFE with a gamma distribution with $\beta=0.1$ and $E_s=-100$. The population state at
506 60,000 generations was saved as a .trees file, at which point the effective population size N_e had
507 stabilized around 100 individuals with around 60,000 segregating deleterious mutations. A
508 neutral burn-in and segregating neutral mutations were then added with recapitation and
509 overlaid mutations according to SLiM 4.0 (Haller et al., 2019). After adding neutral mutations,
510 non-segregating sites (selected or neutral) were added between SNP positions and randomly
511 assigned as either selected (20%) or neutral (5%) to approximate the 0-fold and 4-fold ratios in
512 the empirical *A. lyrata* dataset. A VCF file with 1000 randomly sampled individuals was created
513 from the dataset and used in subsequent analyses with DFE-alpha.

514 To get a baseline accuracy for DFE-alpha, 10 replicates of 100 individuals (the
515 maximum size supported by DFE-alpha) from the simulated dataset were analysed, and the
516 estimation error compared to the known DFE was in each case assessed as the Earth Mover's
517 Distance (see below). To investigate the effects of filtering methods, 15% of the sites in each

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The effect of missing data treatments on DFE¶

We tested three different methods of missing data treatment on two populations of *A. lyrata*, each with three to four different filtering thresholds, to generate a total of 20 VCFs for DFE analysis (Fig. 1a, 2). Results revealed considerable variation in the estimated DFE among these datasets (Table 1). ¶

530 individual in one set of 100 individuals were masked as missing. This dataset was filtered with
531 a) downsampling at a threshold of 85%, b) imputation at a threshold of 85%, or c) subsampling
532 at a threshold of 15%. However, the subsampled dataset retained no 4-fold SNPs in the SFS
533 after filtering, making DFE estimation impossible. We thus instead sampled four replicates of
534 4, 8, 12, 16, 20, 24 and 50 individuals from the 15% missing dataset, and applied subsampling
535 at 100% (i.e. all sites with missing data were excluded). The same sets of sample sizes were
536 then extracted from the downsampled and imputed datasets to compare the accuracy of the
537 different methods while controlling for the effect of sample size. To directly investigate the
538 effect of sample size and SNP number, 10 replicates of 4, 8, 12, 16, 20, 24, 50 and 100
539 individuals were extracted from the datasets with no missing data and analysed with DFE-alpha
540 (Fig. 4a-d).

541 With the DFE associated with the simulated datasets being known, the accuracy of
542 estimated DFE was assessed by comparing them to the known DFE using Earth Mover's
543 Distance (EMD) implemented in the *transport* package in R (Schuhmacher et al., 2019). EMD
544 quantifies the dissimilarity between two distributions as the "work" required to change one
545 distribution to the other, thus taking into account the amount of overlap. In contrast to the widely
546 used Kolmogorov-Smirnov (KS) distance, EMD is not limited by an upper bound, enabling it
547 to more accurately capture substantial differences between distributions. Additionally, EMD is
548 better suited for gauging distances between distributions with long tails. The EMD was
549 evaluated within the range $-10^5 < s < -10^{-3}$ where s represents the selection coefficient for
550 each mutation, in increments of 10^{-3} . Higher EMD values signify a poorer fit between the
551 estimated and true distribution, thus indicating a less accurate result. The EMD values of each
552 dataset was plotted against the number of individuals and SNPs with a regression line to
553 illustrate the relationship.

554 **3 RESULTS**

555 **3.1 The effect of missing-data treatments on DFE in *A. lyrata***

556 *Downsampling.* The datasets downsampled to 50%, 66% and 75% of the genotypes per site
557 retained 105.7M, 99.5M, and 95.0M sites, respectively, for both *A. lyrata* populations (Table
558 1). The Austrian datasets contained 15, 19 and 22 "individuals" and 1.39M, 1.46M and 1.47M
559 SNPs for the three thresholds, while the Norwegian population kept 8, 11, and 12 "individuals"
560 and 374K, 366K and 341K SNPs, respectively. The DFE in the Norwegian datasets differed
561 significantly from that of the Austrian population in that neutral mutations were more frequent
562 (31% vs 33%), while slightly (8% vs 9%) and moderately (10% vs 12%) deleterious mutations were less
563

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Deleted: . The corresponding DFE estimates ranged from 22% to 25% neutral, 11% to 13% slightly deleterious, 15% to 20% moderately deleterious, and 45% to 50% strongly deleterious mutations (Table 1). In

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579 frequent, but the proportion of strongly deleterious mutations was similar (45~51%) (Table 1).
580 Additionally, the impact of filter thresholds from 50% to 75% on the three deleterious groups
581 of mutations in the two populations showed inverse patterns, e.g. strongly deleterious mutations
582 increased with the threshold in the Norwegian population but decreased in the Austrian
583 population. While the estimated DFE varied between populations by 1~10 percentage points
584 under the same method and threshold, it also varied by up to 5 percentage points among the
585 downsampling thresholds within each population.

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586
587 *Imputation.* The imputed datasets retained all individuals (i.e. 29 Austrian and 16 Norwegian
588 individuals), and 103.4M, 97.9M and 86.3M sites at the 70%, 80% and 90% thresholds,
589 respectively. In the Austrian population, 1.69M, 1.63M and 1.44M SNPs were included, while
590 399K, 365K and 341K SNPs in the Norwegian population, at the three thresholds, respectively.
591 Increasing the threshold from 70% to 90% only caused 2~4 percentage points of variation in
592 each category of mutations (Table 1). Across both populations, the DFE were stable among
593 imputation thresholds, with the Austrian population displaying slightly larger variance.

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Deleted: In the Norwegian population, 2.5M, 2.1M and 1.3M SNPs were retained using thresholds 70%, 80% and 90%, respectively. Increasing the threshold from 70% to 90% decreased neutral mutations from 33% to 29%, while the other classes changed less than two percentage points.

594
595 *Subsampling.* In the subsampling trial, we applied four different thresholds, allowing a
596 maximum of 10%, 15%, 20% and 25% missing genotypes per individual. In the Austrian
597 population, a strict threshold of 10% missing data left 8 individuals, 97.4M sites and 844K
598 SNPs in the dataset, while a relaxed 25% threshold preserved all 29 individuals with 55.0M
599 sites and 609K SNPs (Note: increasing the missing rate from 20% to 25% only added one more
600 individual) (Table 1). Increasing the missing threshold from 10% to 25% decreased the
601 estimated neutral mutations from 23% to 20%, and the strongly deleterious mutations from 49%
602 to 32%, while the slightly and moderately deleterious mutations increased from 11% to 17%
603 and from 17% to 30%, respectively. Overall, change the threshold from 10% to 15% induced
604 the largest difference in the DFE of all stepwise increases (3~8 percentage points of difference
605 in all categories).

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606 In the Norwegian population, the dataset filtered with a missing rate of 10% included
607 only 2 individuals with 109.4M sites and 249K SNPs. At this level, the DFE was estimated to
608 7% neutral, 86% slightly deleterious, 6% moderately deleterious and no strongly deleterious
609 mutations. Increasing the threshold to 15% increased the number of individuals to 15, retaining
610 80.0M sites and 172K SNPs, and shifted the DFE to 28% neutral, 8% slightly deleterious, 10%
611 moderately deleterious and 53% strongly deleterious mutation. Further relaxing the missing
612 rate to 20% and 25% included one more individual (16 total) and had little effect on the DFE

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Deleted: 0.9M SNPs. The result for this dataset was very different from the 10% threshold dataset, with the DFE shifting

633 compared to the dataset filtered at 15% (Table 1). Overall, the Austrian population displayed
634 up to 17 percentage points of difference between thresholds, while the Norwegian population
635 displayed up to 79 percentage points of difference when including the ~~dataset~~ filtered at 10%
636 missing data.

637 3.2. The effect of sample size and sites on DFE.

638 ~~We subsampled the~~ Austrian population ~~of A. lyrata~~ into 4, 8, 12, 16, 20 and 24 ~~individual sets,~~
639 ~~each containing 211K, 320K, 357K, 426K, 512K and 557K~~ SNPs, respectively, ~~from~~ the
640 complete dataset of 29 individuals ~~containing 609K~~ SNPs (Fig 3b). We found that decreasing
641 the sample size from 29 to 4 substantially increased the proportion of strongly deleterious
642 mutations from 32% to 45%, while it decreased the proportion of slightly deleterious mutations
643 from 17% to 13% and moderately deleterious mutations from 30% to 20%. Neutral mutations
644 changed only slightly (from 20% to 22%) (Fig. 3a). The partition of DFE remained stable with
645 sample sizes of 8 and upward (≤ 1 percentage points fluctuation). The 95% CIs remained similar
646 and narrow (0.5-4%) in all samples.

647
648 In the second trial, ~~we randomly sampled~~ 1K, 10K, 100K, 1M and 10M sites ~~in the 29~~
649 ~~individuals (with 55.0M sites, 609K SNPs), resulting in 10, 109, 1115, 11.1K, and 111K~~ SNPs,
650 ~~in each dataset,~~ respectively. We found that the DFE estimates became increasingly unstable
651 ~~with~~ decreasing the number of sites: the datasets with fewer than 1M sites (~~11.1K~~ SNPs) showed
652 a large variation in DFE values (8-50 percentage points; Fig. 3b). Notably, a decrease in the
653 number of sites brought a simultaneous increase of the width of the 95% CIs, in a manner not
654 seen when decreasing the numbers of individuals (Fig. 3a vs. 3b). At 1K sites (~~10~~ SNPs), the
655 95% CIs for the three deleterious categories covered 98-100% of the entire range of possible
656 values, indicating low confidence in where the true values lie. At 10K sites (~~109~~ SNPs) the CIs
657 shrunk but were still large, covering between 34-71% of the possible values. On average, each
658 tenfold decrease in the number of sites increased the size of the bootstrapped 95% CIs 2.5 times.

659 In the third trial, we examined the effect of sites in a small sample of 4 individuals.
660 The sites chosen were the same as those in the second trial, although the set of 1K sites included
661 too few SNPs to be evaluated and was not shown in Fig. 3c. The datasets with 10K, 100K, 1M,
662 10M and all 55.0M sites had ~~43, 391, 3821, 38.6K, and 211K~~ SNPs, respectively. At 10K sites,
663 the DFE in the 4-individual set was drastically different from the 29 individuals. Furthermore,
664 the 95% CIs of neutral, and slightly and moderately deleterious mutations increased by 18-81%
665 in the 4-individual relative to the 29-individual dataset. The CIs for strongly deleterious
666 mutations shrank somewhat in the 4-individual dataset but was still large and spanned 66% of

Deleted: Since no individual in this population had a missing rate above 20%, increasing the threshold from 20% to 25% had no effect on the filtered dataset.

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Deleted: In summary, applying different filter methods and thresholds affected the final data matrix size and subsequent DFE estimates. Comparisons of the three missing data treatments suggest that imputation and downsampling produce similar and less variable DFE results among filtering thresholds than does subsampling. ¶

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722 the range of possible values. The DFE estimates at 100K sites and above in 4-individual datasets
723 were very similar (≤ 1 percentage points of difference) to the second trial using 29 individuals
724 (Fig. 3c vs. 3b), but the 95% CIs approximately doubled for the three classes of deleterious
725 mutations.

726

727 **3.3 Accuracy of DFE-alpha in SLiM simulated data**

728 To determine which missing-data treatment and sample sizes produced the least error and thus
729 approximated the true DFE most accurately, we conducted SLiM simulations with a known
730 DFE. The simulation produced a dataset with 1000 individuals and 29,944 SNPs. Using 10
731 replicate samples of 100 individuals, each containing ~15,500 SNPs, the DFE was estimated to
732 between 29~31% neutral, 8-10% slightly deleterious, 10~13% moderately deleterious and
733 48~52% strongly deleterious mutations; the true DFE should be approximately 30% neutral,
734 9% slightly deleterious, 11% moderately deleterious and 50% strongly deleterious mutations,
735 meaning an error of $\pm 1\sim 2\%$ can be expected with this dataset in optimal conditions. The β and
736 E_s parameters of the gamma distributions ranged between 0.097 ~ 0.128 and -276 ~ -33,
737 respectively, yielding error values (EMD $\times 10^7$) between 3.5 ~ 20.5 (Fig. 4e). These values are
738 used as reference for the “maximum” accuracy of DFE-alpha for the simulated dataset.

739 To evaluate the effect of filtering methods, we used four replicates of 4, 8, 12, 16, 20,
740 24 and 50 individuals and excluded all missing sites in each sample, which mimics the effect
741 of subsampling at different thresholds. In order to compare these results to downsampling and
742 imputation, the same sample sizes were extracted from the downsampled and imputed datasets
743 created at 85% threshold from the full dataset. At a sample size of 4 individuals, all three
744 methods performed roughly equally well (average EMD was 33.7, 36.4 and 36.5 for
745 downsampling, imputation and subsampling, respectively. Fig. 4b-d,e, Table S2), but
746 subsampling tended to slightly underestimate the proportion of slightly and moderately
747 deleterious mutations (by up to 5% and 7%, respectively), and overestimate strongly deleterious
748 mutations (by up to 11%). Downsampling gave the most accurate results based on the average
749 EMD across all sample sizes above 8 individuals (Fig. 4b,e). Imputation performed slightly
750 worse in all samples except 8 individuals (Fig. 4c,e). Both downsampling and imputation
751 produced results within 1~3% of the range of the reference set at all sample sizes above 4
752 individuals. Subsampling, however, produced highly variable and noticeably less accurate
753 results even at higher sample sizes (Fig. 4d,e). For example, the 4 replicates of 24 individuals
754 produced EMD values between 3.7 ~ 18.8 for downsampling, 12.3 ~ 47.4 for imputation and
755 31.2 ~ 112.8 for subsampling (Table S2). We found that subsampling produced the most

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756 accurate results at an intermediate sample size (e.g. 16 individuals; EMD from 1.7 to 56.1) and
757 became less accurate at sample sizes where fewer SNPs were retained (e.g. 50 individuals with
758 5 SNPs remaining; Fig. 4b, Table S2).

759 Our simulated data verified the trends observed in the empirical data, showing that
760 increased sample size correlated with lower error in DFE estimates when the number of SNPs
761 is not a limiting factor. In the datasets of 4, 8, 12, 16, 20, 24 and 50 individuals (10 replicates
762 of each) with no missing genotypes, the EMD values were the largest in samples of 4 and 8
763 individuals, stabilized around 12 ~ 24 individuals, and then decreased further in 50 individuals
764 to a level similar to that in the 100 individuals (Fig. 4e). Linear regression in these datasets
765 showed that DFE estimation error (EMD) was negatively correlated with number of individuals
766 ($p = 0.00179$, $R^2 = 0.1182$), and even more strongly correlated with the number of SNPs in the
767 dataset ($p = 6.38 \cdot 10^{-6}$, $R^2 = 0.2311$) (Fig 4f). An even stronger negative correlation between
768 EMD and SNP number was seen when the four replicates of 4 ~ 50 individuals from the
769 downsampled, imputed and subsampled datasets were analysed with a joint linear regression (p
770 = $1.11 \cdot 10^{-9}$, $R^2 = 0.3658$) (Fig 4b). Datasets with few SNPs also displayed larger 95% CIs while
771 the number of individuals had a minor effect on CI size (Fig. 4a-d, Table S2), similar to what
772 was observed in the empirical datasets.

773 In summary, applying different filtering methods and thresholds affected the final data
774 matrix size (number of individuals and SNPs) and subsequent DFE estimates. Imputation and
775 downsampling produced similar and less variable DFE results than subsampling, and
776 downsampling appeared more accurate than imputation for the simulated samples used. Further,
777 higher numbers of individuals and SNPs both increased accuracy of the results, especially at
778 very low sample sizes (4 ~ 8 individuals, <5000 SNPs).

780 **3.4 The effect of population structure on DFE.**

781 The PCA of the 45 samples from Austria and Norway showed a distinct separation of the two
782 populations along PC1 (which explained 24.7% of the total genetic variance), and separation
783 of the Austrian population into four visible clusters along PC2 (which explained 7.3% of the
784 total genetic variance) (Fig. S1). The weighted F_{ST} between the two populations was 0.228,
785 while the F_{ST} among the four Austrian clusters was relatively small as 0.073. To understand the
786 effect of merging genetically distinct populations on the estimated DFE, we created 12 merged
787 populations with contributions of 10 or 15 individuals from Austria and Norway, with three
788 subsets of each population (Fig. 1c). We then calculated the F_{ST} between the contributing
789 subsets to evaluate how the degree of population stratification in a sample affects the joint DFE

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801 estimate. We first examined the DFE in the unmerged replicate samples of 10 and 15 individuals
802 from the two populations. Among the replicates of 10 individuals from the Austrian population,
803 a maximum difference of 2, 3, 7 and 6 percentage points were observed in the neutral, slightly,
804 moderately and strongly deleterious mutations. By comparison, no mutation category varied by
805 more than 2 percentage points in the samples of 15 individuals. Comparably stable DFE
806 estimates were observed in the Norwegian samples, with variation in the range of 0, 2, 3 and 4
807 percentage points for the four categories of mutations in samples of 10 individuals, and ~~less~~
808 ~~than~~ 1 percentage point of a difference among replicates of 15 individuals (Fig. 4a). However,
809 the DFE estimates were markedly different between the two geographical populations, e.g.
810 neutral mutations shifted up by an average of 9 percentage points while the slight and moderate
811 mutations shifted downwards in Norway compared to Austria. The estimated proportions of
812 strongly deleterious mutations were similar in the two populations.

813 With this population-specific DFE in mind, we then examined the differences between
814 the merged samples and their respective contributing single population subsets. In most cases,
815 the estimated DFE values for the merged samples were in-between the DFE estimates of the
816 contributing subsets, but not always perfectly intermediate (Fig. 5a). ~~The estimated weighted~~
817 ~~F_{ST} values between the pairs of contributing subsets ranged from 0.218 to 0.263 (mean F_{ST}~~
818 ~~between 0.085 and 0.131). These estimates are largely in line with previous studies, where~~
819 ~~mean F_{ST} across European populations of *A. lyrata* ranges between 0.06-0.09 (Marburger et al.,~~
820 ~~2019). Plotting the weighted F_{ST} against the estimated DFE in the merged populations showed~~
821 ~~an apparent relationship (Fig. 5b).~~ Using linear regression, F_{ST} was correlated with the
822 proportion of slightly ($R = -0.61$, $p = 0.037$), moderately ($R = -0.60$, $p = 0.038$) and strongly
823 deleterious mutations ($R = 0.66$, $p = 0.02$), but not with that of neutral mutations ($p = 0.17$).
824 These results show that population structure had a significant effect on the deleterious portion
825 of the DFE, with higher F_{ST} potentially driving up the estimated proportion of strongly
826 deleterious mutations and reducing the estimates of the less deleterious classes.

827 **4 DISCUSSION**

828 **4.1 Methods of missing data treatment affect DFE results**

829 ~~Missing-data treatment is the first step in any genomics analyses. Using simulated data with~~
830 ~~known DFE we were able to evaluate the accuracy of different filtering methods in recovering~~
831 ~~the true DFE. We found the dataset with no missing data produced the most accurate result,~~
832 ~~followed by downsampling, then imputation, and then subsampling. The number of SNPs in~~
833 ~~the downsampled and imputed datasets were similar in all samples, suggesting that any~~

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849 difference in performance between the two methods is likely due to imputation affecting the
850 shape of the SFS in a non-random manner. The assumption that deleterious mutations appear
851 as low-frequency alleles in the SFS, in combination with the relatively small sample sizes used
852 in the tests, makes an SFS-based analysis highly reliant on those low-frequency categories,
853 especially singleton SNPs. Low frequency alleles thus display much higher error rates than
854 higher-frequency alleles in imputation procedures (Pook et al., 2020).

855 Filtering with subsampling produced the least accurate estimates on average. Since
856 increasing the number of individuals in the subsampled dataset decreases the number of sites,
857 this filtering method's performance is thus affected by sample size in two ways, both the number
858 of individuals and the number of SNPs available. This effect is expected to be especially strong
859 in datasets where the distribution of missing data is random (as was the case in our simulated
860 datasets), where a highly dissimilar pattern of missing data across individuals excludes a large
861 number of sites by subsampling. This pattern was not as strong in the empirical datasets where
862 the missing data across individuals was more similar. Thus intermediate sample sizes of
863 individuals are preferable for this method.

864 The array of tested filtering thresholds on the empirical datasets corroborated the trend
865 and conclusions drawn from the simulated datasets. The empirical datasets proved to be more
866 sensitive to minor changes in filtering thresholds as even slight adjustments resulted in
867 significantly different outcomes in some cases. The DFE estimates in the subsampled datasets
868 were unpredictable, both within and among populations. This is most likely a result of
869 substantial downsizing of the data matrix, since the total number of sites and SNPs were reduced
870 by 50–90% in the subsampled datasets compared to the other two methods. Downsampling and
871 imputation produced results with similar levels of variation across the different thresholds. With
872 the simulation results in mind, it could be argued that both methods are be equally valid in this
873 case, and the choice between them might depend on other conditions and computational
874 resources. As a general rule, we recommend filtering data with several thresholds to obtain an
875 overview of the variability produced by each method. This is especially important because the
876 95% CIs do not provide information about whether the filtered and subsampled dataset is
877 representative of the initial population and, as we show in this study, the differences among
878 subsets of samples from the same population can be significant.

879 A cursory review of recently published DFE estimation studies shows that
880 downsampling is the most frequently used of the three methods tested here (see Castellano et
881 al., 2019; Chen et al., 2020; Gossmann et al., 2010; Liang et al., 2022; Takou et al., 2021). This
882 is not surprising, since downsampling is considerably faster than imputation, yet retains more

Deleted: However, as we do not know the true DFE in empirical samples, it is impossible to know which filter method and settings produce results that are closer to the true value. We advise that future research use the method that preserves the largest amount of data and produces the least variable results with the same data set. In this regard, imputation seems to be the most reliable method of processing missing data, especially for large datasets – although downsampling would come a close second.

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906 data than subsampling. Imputation methods require high quality datasets from the outset to be
 907 able to make reliable predictions; datasets with high rates of missing sites and low levels of
 908 genome-wide linkage disequilibrium are not ideal for this treatment. With low levels of
 909 genome-wide linkage disequilibrium, the presence/absence of any given SNP is mostly
 910 uncorrelated with the presence/absence of any other SNP, meaning that there are no patterns of
 911 linkage disequilibrium among sites from which imputation can accurately predict the state of a
 912 missing site. In such cases, downsampling might be a better choice. With the current rate of
 913 improvement in both genome-wide sequence data and computing power, however, we predict
 914 an increasing popularity of imputation as a data processing method in DFE estimation and other
 915 population genomics analyses. We recommend prefacing any missing-data treatment with an
 916 analysis of the prevalence of missing sites and the level of linkage disequilibrium to determine
 917 whether imputation is the appropriate method for each dataset.

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919 **4.2 Very small sample sizes skew the estimated DFE**

920 A review on DFE estimated in 139 plant and animal species (Chen et al., 2017), each with
 921 between 2–50 chromosomes sampled, shows very different DFE distributions. We evaluated
 922 the effects of the number of sampled individuals on the estimated DFE when the number of
 923 sites was not a limiting factor. We found that DFE estimated from few individuals (<8) were
 924 strongly skewed compared to larger sample sizes. In simulated datasets with no missing data,
 925 the accuracy of the estimated DFE was highest in the largest sample (100 individuals) and
 926 lowest in the smallest samples (4 and 8 individuals), and the samples with >8 individuals
 927 markedly improved DFE estimates. Similarly, DFE estimates based on 4 individuals produced
 928 the least accurate results using both downsampling and imputation for missing-data treatment.

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929 In the empirical trials, DFE estimates between random sets of 4 individuals were rather
 930 unstable in the Austrian population. In the Norwegian dataset subsampled at 10% that kept only
 931 2 diploid individuals, the proportion of slightly deleterious mutations was greatly overestimated
 932 compared to that of the full population size. Results stabilized with a sample size of 8 or more,
 933 which is consistent with the findings from the simulated datasets. This suggests that a relatively
 934 small number of individuals is needed for reliable DFE estimates when there are many sites
 935 available, but that very limited sample sizes increases the risk of producing non-representative
 936 results. We thus deem the potential effects of low sample size to be alarming due to their
 937 unpredictable and stochastic nature, and caution against using sample sizes below 4 diploid
 938 individuals (8 haploids).

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4.3 Limited sites cause high variability in DFE results

Reducing the number of sites resulted in highly variable and unpredictable DFE estimates even with larger sample sizes. Overall, the negative correlation was observed between the number of SNPs and EMD values in the simulated datasets indicates that the accuracy of SFS-based DFE estimation is limited by the number of SNPs available. This trend was also observed in the empirical data, where estimates based on 1M, 10M and 55M sites in 29 individuals all looked similar, but using 1K ~ 10K sites (59 ~ 571 SNPs) produced highly dissimilar results, demonstrating the importance of having a sufficient number of sites and SNPs for reliable SFS-based analyses. The DFE is estimated from SFS, i.e. the distribution of SNPs of different frequencies in the population. Thus, the number and specific subset of SNPs directly affect the resolution to which we can estimate the shape of the DFE. This would explain why the 95% CIs increased in size as the number of sites decreased. At 1K ~ 10K sites, the confidence intervals spanned the entire range of possible values for several of the mutational categories (Fig. 3b). For these datasets, we are therefore left with no confidence that our predicted DFE is close to the true DFE. If the CIs are ignored, the very different DFE estimates from subsets of the same dataset could lead to different interpretations of the selection pressures acting on the population. This result illustrates a clear type 1 error; the estimated DFE from our samples of 1K, 10K and 100K sites are not representative of the full sites and produce incorrect inferences that imply differences in the underlying DFE, despite being random subsets of the same dataset.

Based on both the empirical and simulated trials, we conclude that DFE estimates of DFE become stochastic and unpredictable, with very small number of sites/SNPs, and accuracy is expected to increase significantly with the number of SNPs included; at least 5K SNPs are required to obtain reliable DFE estimates, using DFE-alpha.

4.4 Population structure may skew DFE estimates

By combining samples from the Austrian and Norwegian populations into merged populations, we were able to see how the composition of populations affects DFE estimates. One trend was immediately clear: the estimated proportion of strongly deleterious mutations was higher in the merged populations than in the contributing single population subsets. A high F_{ST} may skew the DFE towards higher estimated proportions of strongly deleterious mutations and lower proportions of slightly and moderately deleterious mutations. This correlation may not be conclusive, but it indicates that population structure can indeed affect DFE and should be taken into consideration when performing these analyses at a species level. Studies on DFE often include multiple or combined populations to gain a global estimate that characterizes the

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Deleted: In most cases, the other three mutational categories displayed mostly intermediate values between the two contributing populations. Our evaluation of population differentiation on DFE in the merged samples further illustrates that a

1036 organism or species (Chen et al., 2017; Härmälä & Tiffin, 2020; Slotte et al., 2010; Zhao et al.,
1037 2020). We cannot presently state that pooled samples will always skew the DFE distribution,
1038 but it is advisable to estimate the DFE separately in individual populations, as well as from
1039 pooled samples to evaluate any deviations caused by pooling that might inform conclusions
1040 drawn from the results. A recent study developed a joint DFE approach that enables the analysis
1041 of pairs of populations (Huang et al., 2021), which could be practical in examining variance of
1042 DFE among populations.

1043 **5 CONCLUSION**

1045 Accurate estimation of DFE from genomic data hinges on several factors, including the number
1046 of sampled individuals, the availability of sites and SNPs, and the approach employed to address
1047 missing data. Our study, which utilized both empirical data and forward simulations, explored
1048 all these aspects and offers guidance for experimental design of DFE estimation studies. We
1049 found that downsampling is a dependable method of handling missing data, though it may still
1050 impact the DFE to some extent. Imputation, while generally accurate, may be less suitable for
1051 small samples (<100 individuals, <10K SNPs) or when genome-wide linkage disequilibrium is
1052 very low (as is often the case with highly outbreeding species). We demonstrated that DFE
1053 estimates derived from datasets with less than four diploid individuals or less than 5K SNPs
1054 may be unreliable, due to the risk of sampling error and the limited information in the SFS.
1055 Furthermore, strong population structure within samples can potentially skew DFE estimates.

1056 More advanced methods of DFE estimation employ an unfolded SFS, where each SNP
1057 is categorized as ancestral or derived based on an outgroup reference genome. While model
1058 species can benefit from these sophisticated techniques, most studies must still rely on methods
1059 utilizing the folded SFS, and frequently deal with limited sample sizes. Given the extensive
1060 body of previously published work employing folded SFS, it is imperative to be able to
1061 understand the expected accuracy of DFE estimates in comparative analyses. This study
1062 highlights the factors that should be considered when interpreting DFE estimates, thereby
1063 enhancing the reliability and relevance of future research.

1064 **ACKNOWLEDGEMENTS**

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1067 Swedish National Infrastructure for Computing (SNIC), through the High Performance
1068 Computing Centre North (HPC2N). This study was supported by grants from the Swedish
1069 Research Council (VR) and T4F program to XRW. The authors declare no conflict of interest.

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The distribution of fitness effects of new mutations shapes the tempo and mode of molecular and organismal evolution. The ability to accurately estimate

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Deleted: relative fitness of mutations and their frequency distribution under various mating, selection and demographic conditions would be valuable paths to follow.

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We thank professor Barbara Giles for valuable comments and linguistic editing.

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AUTHOR CONTRIBUTION

WZ and XRW designed the empirical study. All authors contributed to designing the simulation study. BH provided support for simulations in SLiM 4.0. BA and WZ performed empirical data analyses. ÅB provided statistical advice. BA, WZ and XRW wrote the manuscript draft. All authors contributed to the revision of the manuscript.

DATA AVAILABILITY

All sequencing data are retrieved from the NCBI SRA database with accession numbers listed in Supplemental Table 1. Procedures associated with the SLiM simulations are provided to GitHub repository at <https://github.com/beaangelica/DFE-filtering>.

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1248 **Figure legends**

1249 **Figure 1: Experimental design**

1250 We performed three sets of tests to understand their potential influence on the estimated DFE:
1251 a) three procedures of missing-data treatment, b) the number of individuals and sites used, and
1252 c) population structure. Each box represents a derived dataset, with the number of individuals
1253 shown on top and nucleotide sites below. The study involved two populations of *Arabidopsis*
1254 *lyrata* from Austria and Norway. We created merged populations with subsets of individuals
1255 from Austria and Norway as specified on the left of each merged boxes (c, greyed out). The
1256 estimated DFE of the merged population are compared to that of the contributing populations.

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1258 **Figure 2: Methods of missing-data treatments for SFS based analyses**

1259 Illustration of the different steps involved in the three missing-data filtering methods examined
1260 in this study. Each box corresponds to an individual's genotype at a site, and missing boxes
1261 represent missing data for a genotype. In downsampling, step 1 excludes sites at which data is
1262 missing in more than a prescribed fraction of individuals (e.g. 25%), while step 2 samples
1263 genotypes without replacement from the remaining data at each site. In imputation, as in
1264 downsampling, step 1 excludes sites with missing rate more than a prescribed fraction, while
1265 step 2 imputes (fills in) missing data. In subsampling, step 1 excludes individuals with missing
1266 data in more than a prescribed fraction of sites, while step 2 excludes all sites with missing data.

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1268 **Figure 3: Effects of number of individuals and sites on DFE**

1269 DFE estimated from *Arabidopsis lyrata*. a) random samples of 4, 8, 12, 16, 20 and 24 of the 29
1270 individuals of the Austrian population with 55M sites; b) all 29 individuals, c) a random sample
1271 of 4 individuals with 1K, 10K, 100K, 1M, 10M and 55M sites. The complete DFE is represented
1272 as percentage contribution of each of four categories of mutations: *neutral* (blue), *slightly*
1273 *deleterious* (yellow), *moderately deleterious* (orange) and *strongly deleterious* (red). The DFE
1274 for each sample size is represented in two ways: on the left as stacked estimated percentages of
1275 the four categories of mutations, and on the right as the estimated percentage of each category
1276 of mutations (black bars and light areas) together with the 95% CIs (darker coloured areas).

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1278 **Figure 4: The accuracy of DFE estimations by manipulating SLiM simulated dataset**

1279 DFE estimates and 95% CIs for 4, 8, 12, 16, 20, 24 50, and a maximum of either 85 (in
1280 downsampling) or 100 (in the other cases) individuals, with either a) no missing data, or 15%
1281 missing data per individual and filtered with either b) downsampling, c) imputation or d)

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1289 subsampling. e) DFE estimation error, as represented by Earth Mover's Distance (EMD), in
1290 different sample sizes without missing data (black, 10 replicates (n) per sample size), or with
1291 15% missing data and filtered with either downsampling (green), imputation (red) or
1292 subsampling (yellow), in four replicates each. f) DFE estimation error in samples plotted against
1293 SNP number, in datasets without missing data (black) as well as with missing-data filtered by
1294 downsampling (green), imputation (red) or subsampling (yellow). Linear regression lines for
1295 the no missing data (black) and for all of the filtered datasets combined (brown) are displayed
1296 to show the trend of EMD over SNP number in the two groups. Datasets without missing data
1297 include 10 replicates of 4 ~ 100 individuals, while four replicates of 4 ~ 50 individuals are
1298 included for the missing-data filtered datasets.

1299

1300 **Figure 5: Effect of population structure on DFE**

1301 a) The estimated DFE of the Austrian (dark dots) and Norwegian (light dots) samples of
1302 *Arabidopsis lyrata*, compared to merged samples (solid lines) containing both groups in
1303 different combinations. The relative sample size from each population is listed along the
1304 horizontal axis (bottom), as well the name of each of three replicates (top). b) Linear regression
1305 of the estimated proportion of each of the four mutational categories of the DFE over the F_{ST}
1306 between the merged samples, with 95% confidence intervals shown in shaded areas.

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1309 **SUPPORTING INFORMATION**

1310 **Table S1.** Sequence information of the *Arabidopsis lyrata* samples included in this study.

1311 **Table S2.** Accuracy of DFE estimations for different missing-data treatments and sample sizes
1312 from the SLiM simulated dataset.

1313 **Figure S1.** Principal component analysis in the 45 individuals of *Arabidopsis lyrata* sampled
1314 from Austria and Norway, based on 3,921,575 SNPs.

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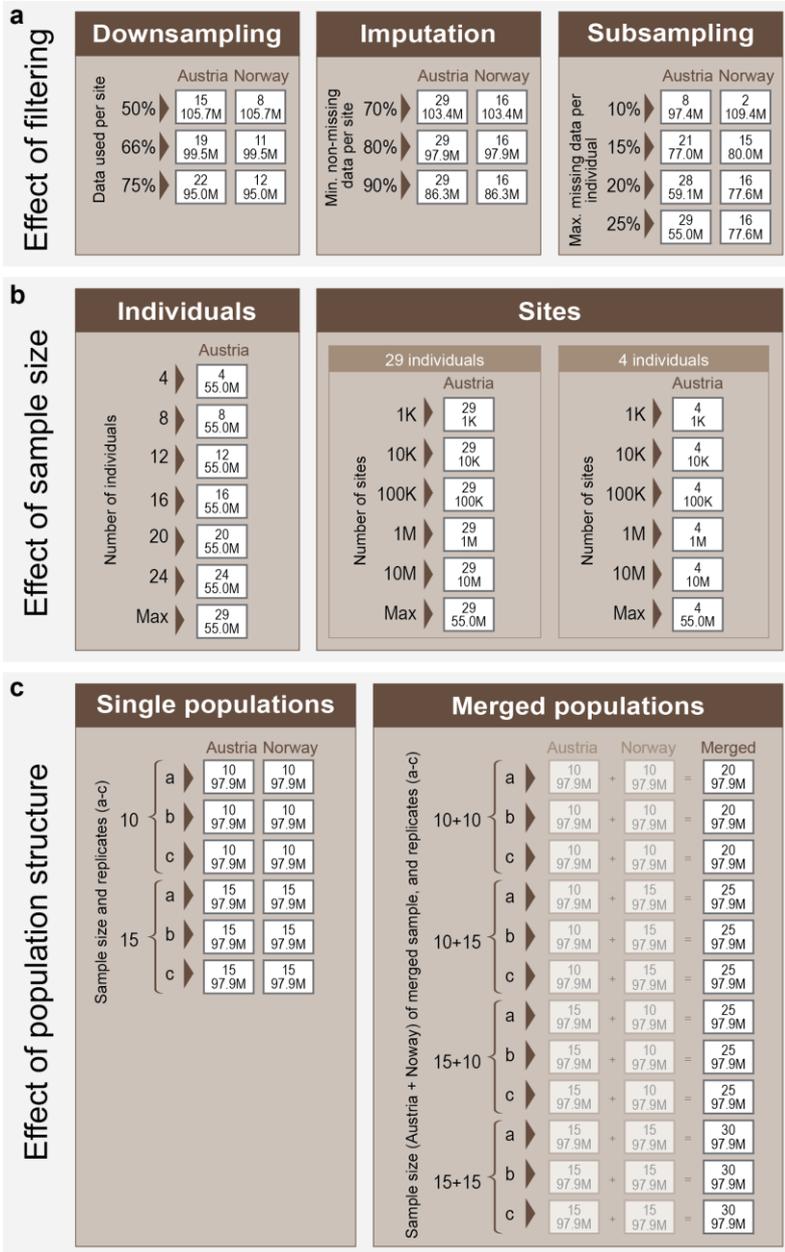
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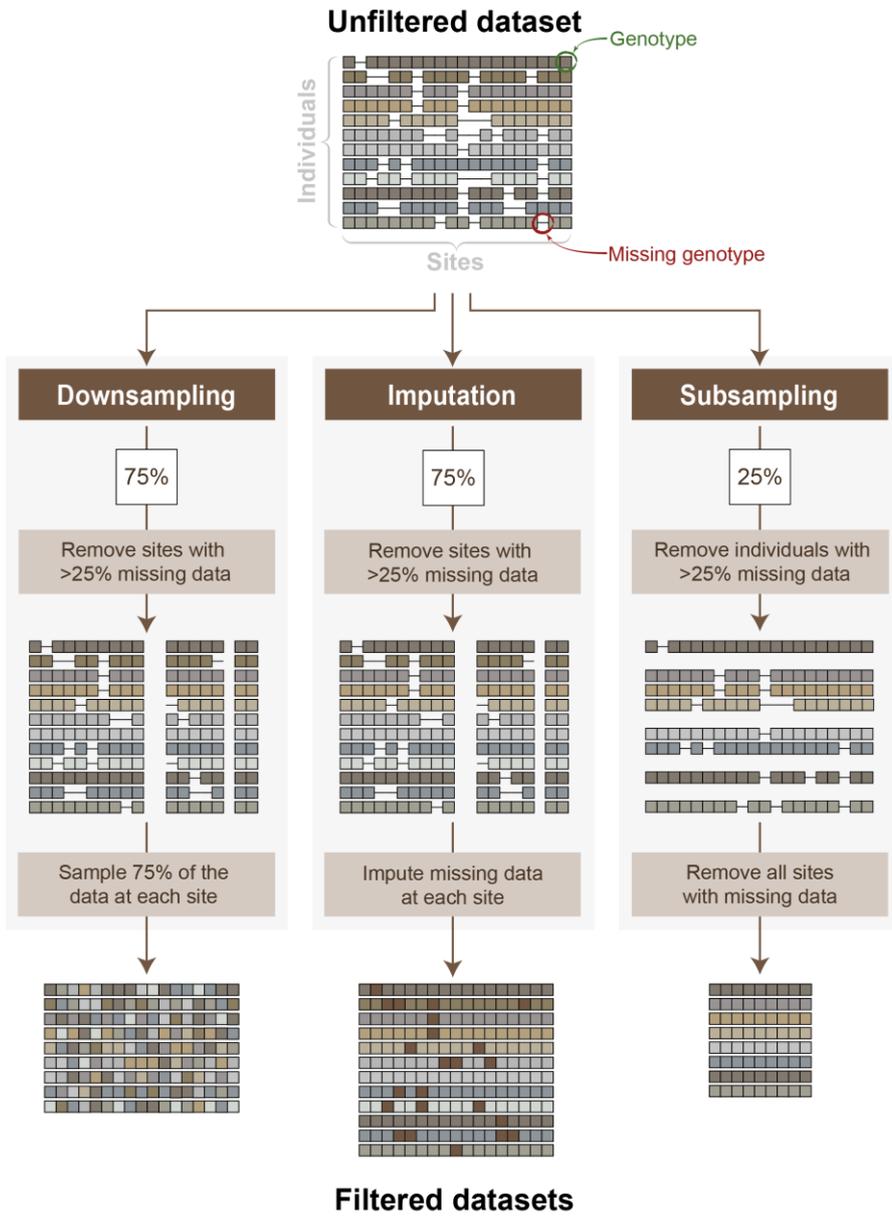
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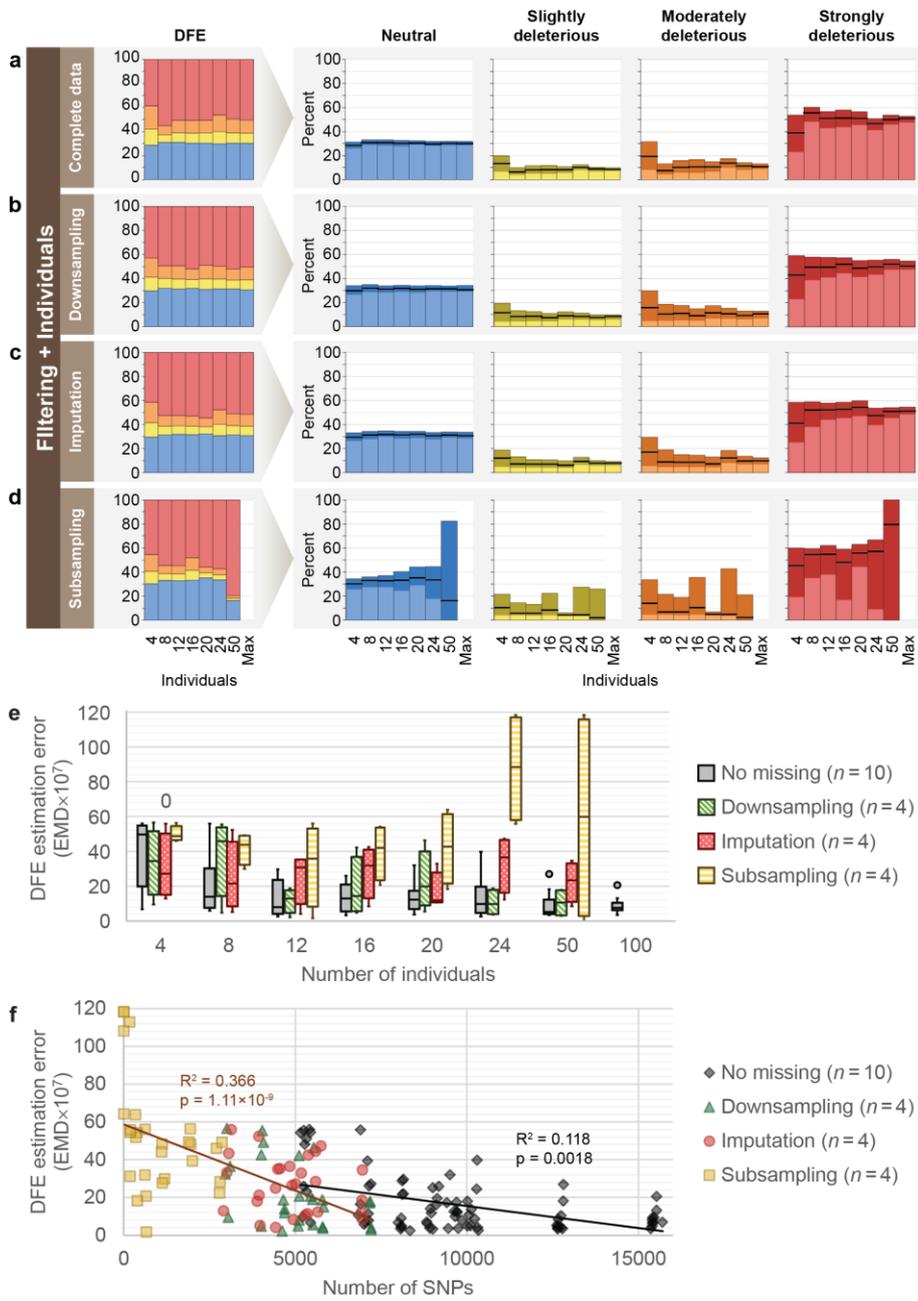
1319 **Figure 1.**



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1325 **Figure 4.**



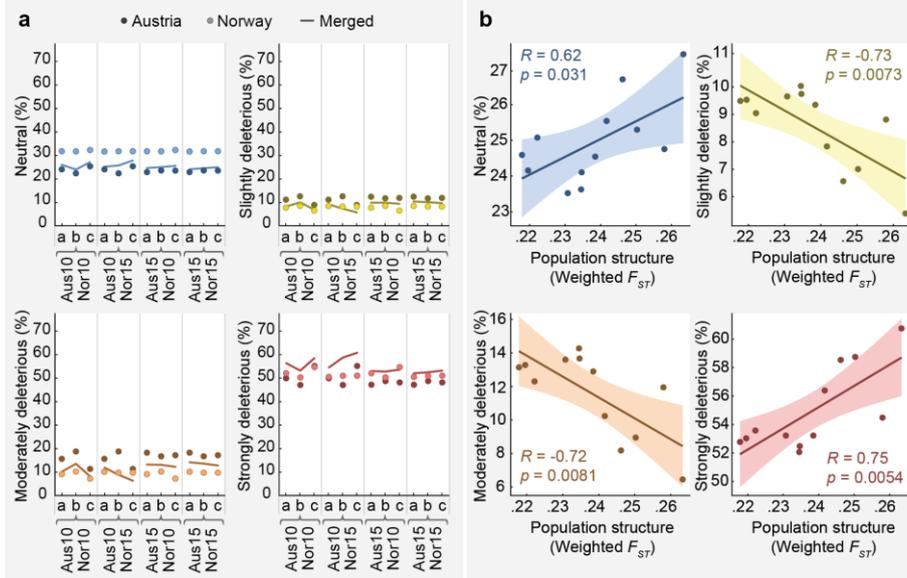
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1330 **Figure 5.**



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Table 1. The estimated DFE using downsampling, imputation and subsampling procedures in Austrian and Norwegian populations of *A. lyrata*. For downsampling, thresholds show the percentage of data retained at each locus. Imputation thresholds signify the data quality (inverse of the max missing rate) of the individuals included in the dataset prior to imputation. Subsampling thresholds signify the max missing rates per individual.

	Filtering method	Individuals	Total sites	0-fold sites	4-fold sites	SNPs in SFS	DFE [95% CI]: % of mutations with $-N_e s$ values of:				
							[0, 1]	(1, 10]	(10, 100]	> 100	
Austria	Downsampling	50%	15	105,746,755	20,081,506	4,532,827	↓389,235	24.63 [24.53–24.73]	10.59 [10.47–10.72]	15.11 [14.87–15.34]	49.68 [49.37–49.96]
		66%	19	99,518,927	19,796,632	4,465,916	↓455,146	23.26 [23.15–23.35]	11.73 [11.61–11.80]	17.53 [17.30–17.70]	47.48 [47.26–47.80]
		75%	22	95,023,967	19,576,647	4,410,244	↓472,536	21.76 [21.69–22.95]	12.91 [11.45–13.00]	20.30 [17.07–20.48]	45.03 [44.75–48.57]
	Imputation	70%	29	103,436,893	19,988,452	4,509,075	↓686,431	23.08 [22.97–24.04]	12.39 [11.26–12.51]	18.87 [16.47–19.12]	45.66 [45.35–48.25]
		80%	29	97,909,623	19,724,874	4,444,971	↓625,688	22.67 [22.57–22.77]	12.12 [11.99–12.22]	18.44 [18.17–18.65]	46.77 [46.50–47.12]
		90%	29	86,272,541	19,088,682	4,267,939	↓437,770	20.21 [20.09–20.33]	13.56 [13.46–13.68]	22.24 [21.99–22.52]	43.99 [43.69–44.27]
	Subsampling	10%	8	97,383,774	19,593,039	4,364,309	↓43,938	22.96 [22.80–23.28]	11.21 [10.60–11.43]	16.61 [15.39–17.05]	49.22 [48.61–50.79]
		15%	21	76,996,896	18,026,562	3,900,662	↓74,047	19.78 [19.60–19.90]	14.68 [14.50–14.87]	24.81 [24.40–25.28]	40.74 [40.16–41.24]
		20%	28	59,099,749	14,532,777	3,066,767	↓662,641	20.11 [19.41–20.29]	16.87 [16.62–17.62]	29.21 [28.62–31.15]	33.81 [31.81–34.50]
25%		29	54,974,337	13,445,210	2,824,524	↓609,256	20.31 [20.10–20.56]	17.29 [16.96–17.56]	29.93 [29.16–30.56]	32.47 [31.72–33.38]	
Norway	Downsampling	50%	8	105,746,755	20,081,506	4,532,827	↓74,403	33.13 [32.78–33.45]	9.40 [7.96–10.17]	12.06 [9.86–13.30]	45.41 [43.60–48.80]
		66%	11	99,518,927	19,796,632	4,465,916	↓66,254	32.05 [31.58–32.32]	7.91 [7.42–9.95]	9.86 [9.13–13.07]	50.17 [45.39–51.36]
		75%	12	95,023,967	19,576,647	4,410,244	↓41,308	30.91 [30.70–31.15]	8.11 [7.72–8.67]	10.24 [9.64–11.10]	50.74 [49.38–51.59]
	Imputation	70%	16	103,436,893	19,988,452	4,509,075	↓99,078	32.83 [32.58–33.04]	6.51 [6.20–6.96]	7.80 [7.37–8.44]	52.86 [51.89–53.62]
		80%	16	97,909,623	19,724,874	4,444,971	↓65,494	31.62 [31.33–31.89]	6.64 [6.26–7.20]	8.04 [7.49–8.85]	53.71 [52.54–54.49]
		90%	16	86,272,541	19,088,682	4,267,939	↓68,958	29.30 [29.04–29.68]	8.06 [7.09–8.41]	10.27 [8.79–10.83]	52.38 [51.49–54.50]
	Subsampling	10%	2	109,442,991	20,192,673	4,555,968	↓48,706	7.26 [7.00–7.48]	86.62 [86.37–86.99]	6.12 [5.89–6.30]	0.00 [0.00–0.00]
		15%	15	79,983,985	18,525,720	4,136,084	↓79,342	28.20 [27.89–28.57]	7.97 [7.46–8.23]	10.22 [9.42–10.64]	53.60 [53.01–54.72]
		20%	16	77,586,760	18,343,099	4,091,607	↓71,711	28.36 [27.78–28.69]	7.55 [7.02–8.83]	9.56 [8.76–11.61]	54.53 [51.62–55.79]
25%		16	77,586,760	18,343,099	4,091,607	↓71,711	28.36 [27.78–28.69]	7.55 [7.02–8.83]	9.56 [8.76–11.61]	54.53 [51.62–55.79]	

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Table S1. Sequence information of the *Arabidopsis lyrata* samples included in this study. Source codes are NCBI accession IDs.

Population	ERRS	Coverage (Mb, >=5x)	Mean depth (>=5x)	Median depth (>=5x)	Size (reads)
Norway	ERR3397904	147.06	25.71	16	53201441
Norway	ERR3397905	144.61	21.83	14	52859026
Norway	ERR3397906	143.18	22.77	13	54633798
Norway	ERR3397907	143.9	22.27	14	53839619
Norway	ERR3397908	146.44	23.74	15	59507152
Norway	ERR3397909	146.45	23.69	15	59465532
Norway	ERR3397910	145.45	24.72	14	62069317
Norway	ERR3397911	148	26.05	15	66516823
Norway	ERR3397912	145.1	23.77	14	59254019
Norway	ERR3397913	143.16	21.8	13	53951341
Norway	SRR5124977	151.64	70	38	127927590
Norway	SRR5124983	144.92	33.26	22	59142085
Norway	SRR5124985	135.63	22.97	13	34814708
Norway	SRR5124997	153.66	55.35	35	102114289
Norway	SRR5124998	149.93	49.79	30	88534336
Norway	SRR5124999	139.45	27.54	16	42830997
Austria	ERR3514864	130.18	17.02	11	20727733
Austria	ERR3514865	141.4	23.75	15	31042548
Austria	ERR3514866	140.31	21.29	15	27625035
Austria	ERR3514869	156.87	49.12	37	72515100
Austria	ERR3514870	145.82	25.47	18	34493579
Austria	ERR3514871	147.85	26.79	19	36994924
Austria	ERR3514872	130.12	17.95	11	22086024
Austria	ERR3514873	136.53	19.89	13	25584056
Austria	ERR3514874	141.42	26.26	16	34646984
Austria	ERR3514875	128.41	16.94	10	20457660
Austria	ERR3514876	148.58	29.17	22	39961518
Austria	ERR3514877	130.4	16.15	11	19708718
Austria	ERR3514878	155.98	42.5	33	62109925
Austria	ERR3514879	141.14	21.85	14	29584688
Austria	ERR3514880	153.3	38.58	27	54727972
Austria	ERR3514883	130	19.1	11	23003063
Austria	ERR3514884	130.2	17.75	11	21314626
Austria	ERR3514885	141.2	27.63	16	37910237
Austria	ERR3514886	124.05	15.02	10	17565928
Austria	ERR3514887	144.2	26.05	17	35825064
Austria	ERR3514888	142.67	26.2	17	34966847
Austria	ERR3514889	141.14	24.65	16	32554512
Austria	ERR3514892	138.26	19.26	14	24924196
Austria	ERR3514893	142.71	23.43	16	30871081
Austria	ERR3514895	148.06	30.95	20	42713949
Austria	ERR3514896	149.81	31.97	23	44511357
Austria	ERR3514897	135.69	22.12	13	27521061
Austria	ERR3514898	145.12	32.64	21	44216326
Austria	ERR3514899	144.58	29.89	19	39745319

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Table S2: Number of sites and SNPs in different simulated datasets, together with their respective estimated mean (E_s) and shape (β) parameters of the DFE. Earth Mover's Distance (EMD) signifies the accuracy of each estimated gamma distribution of DFE to the known DFE used in the simulation (shape β : 0.1, mean E_s : -100). Lower EMD values indicate a closer fit to the known DFE.

Filtering method	Individuals	Sites in VCF after filtering	Sites in SFS (min – max)	SNPs in SFS (min – max)	β		E_s		EMD (10^7)		
					(Median)	(min – max)	(Median)	(min – max)	(Median)	Mean	(min – max)
No missing data (in 10 replicates)	4	50,000,000	12,162,648 – 12,162,869	5,156 – 5,448	0.0902	[0.0500 – 0.1675]	-682	[-6.36×10 ⁶ – -4]	49.7	38.5	[6.7 – 56.5]
	8	50,000,000		6,840 – 7,205	0.1064	[0.0500 – 0.1318]	-127	[-5.19×10 ⁶ – -21]	13.9	20.8	[5.8 – 56.5]
	12	50,000,000		8,002 – 8,342	0.1077	[0.0865 – 0.1326]	-103	[-1,044 – -18]	7.9	13.3	[2.5 – 30.5]
	16	50,000,000		8,832 – 9,182	0.1043	[0.0900 – 0.1308]	-148	[-714 – -22]	12.9	13.3	[3.2 – 26.5]
	20	50,000,000		9,420 – 9,859	0.1044	[0.0843 – 0.1220]	-134	[-1,293 – -37]	12.3	13.0	[3.7 – 32.5]
	24	50,000,000		10,019 – 10,336	0.1051	[0.0792 – 0.1291]	-126	[-2,773 – -24]	9.8	13.2	[2.6 – 40.5]
	50	50,000,000		12,617 – 12,818	0.1096	[0.0874 – 0.1235]	-94	[-821 – -36]	5.3	8.9	[3.4 – 27.5]
	100	50,000,000		15,357 – 15,702	0.1138	[0.0972 – 0.1280]	-79	[-276 – -33]	7.5	8.9	[3.5 – 21.5]
Downsampling (in 4 replicates)	4	28,420,524	6,911,406	2,959 – 3,099	0.1253	[0.0500 – 0.1411]	-39	[-1.23×10 ⁷ – -11]	34.5	33.7	[9.4 – 57.5]
	8	28,420,524		4,010 – 4,075	0.0724	[0.0500 – 0.1001]	-8,392	[-3.15×10 ⁶ – -147]	45.9	38.0	[4.8 – 55.5]
	12	28,420,524		4,621 – 4,694	0.0999	[0.0919 – 0.1162]	-198	[-420 – -52]	13.0	11.7	[2.2 – 19.5]
	16	28,420,524		5,085 – 5,122	0.0945	[0.0777 – 0.1082]	-336	[-3,711 – -80]	14.4	19.0	[4.9 – 42.5]
	20	28,420,524		5,446 – 5,539	0.0907	[0.0727 – 0.1096]	-456	[-6,829 – -78]	19.9	22.9	[5.5 – 46.5]
	24	28,420,524		5,795 – 5,827	0.0981	[0.0913 – 0.1030]	-229	[-417 – -136]	9.8	10.5	[3.7 – 19.5]
	50	28,420,524		7,165 – 7,216	0.0992	[0.0929 – 0.1088]	-248	[-391 – -88]	10.6	10.5	[3.0 – 18.5]
	85 (n=1)	28,420,524		8,323	0.1045		-139		4.0	4.0	
Imputation (in 4 replicates)	4	28,420,524	6,911,406	2,907 – 3,136	0.1140	[0.0500 – 0.1498]	-137	[-5.64×10 ⁶ – -7]	38.3	36.4	[12.9 – 56.5]
	8	28,420,524		3,892 – 3,976	0.0998	[0.0642 – 0.1171]	-293	[-54,893 – -32]	19.6	24.2	[5.2 – 52.5]
	12	28,420,524		4,426 – 4,554	0.0848	[0.0815 – 0.1079]	-1,201	[-1,762 – -84]	30.0	24.9	[4.1 – 35.5]
	16	28,420,524		4,840 – 5,017	0.0866	[0.0794 – 0.1111]	-811	[-1,953 – -63]	26.8	24.6	[8.3 – 36.5]
	20	28,420,524		5,179 – 5,384	0.0894	[0.0760 – 0.0981]	-819	[-3,850 – -226]	22.2	24.4	[10.5 – 43.5]
	24	28,420,524		5,592 – 5,750	0.0793	[0.0729 – 0.1155]	-2,976	[-8,094 – -50]	36.5	33.2	[12.3 – 47.5]
	50	28,420,524		6,931 – 6,958	0.0948	[0.0814 – 0.1132]	-334	[-1,642 – -65]	15.4	18.5	[8.5 – 35.5]
	100 (n=1)	28,420,524		8,396	0.0986		-247		11.6	11.6	
Subsampling (in 4 replicates)	4	26,099,661	6,347,733	2,707 – 2,870	0.1279	[0.0733 – 0.1518]	-23	[-11,804 – -6]	37.2	36.5	[22.4 – 49.5]
	8	13,629,024	3,313,984	1,897 – 2,003	0.0702	[0.0500 – 0.0737]	-11,337	[-8.96×10 ⁶ – -2,657]	48.7	48.3	[39.4 – 56.5]
	12	7,109,386	1,730,591	1,107 – 1,194	0.1040	[0.0696 – 0.1366]	-2,386	[-10,280 – -18]	37.0	37.5	[27.7 – 48.5]
	16	3,713,156	902,526	624 – 661	0.1109	[0.0782 – 0.1618]	-59	[-1,223 – -3]	26.3	27.6	[1.7 – 56.5]
	20	1,938,476	470,900	348 – 402	0.0907	[0.0500 – 0.1938]	-35,852	[-795,504 – -2]	53.1	47.1	[18.3 – 64.5]
	24	1,011,970	246,323	174 – 199	0.0540	[0.0500 – 0.5804]	-362,712	[-3.23×10 ⁶ – 0]	55.1	63.6	[31.2 – 113.5]
	50	14,863	3,737	3 – 5	0.5172	[0.0500 – 0.5227]	0	[-2.16×10 ¹² – 0]	113.2	102.3	[64.2 – 118.5]

Figure S1. Principal component analysis in the 45 individuals of *Arabidopsis lyrata* sampled from Austria and Norway, based on 3,921,575 SNPs.

