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Identification of inter- and intra-species variation in cereal grains through geometric morphometric analysis, and its resilience under experimental charring.

Running title

Geometric morphometric analysis of cereal grains

Authors

Vincent Bonhomme[1,2], Emily Forster[3], Michael Wallace[3], Eleanor Stillman[1], Michael Charles[4], Glynis Jones[3]

Affiliations

[1]School of Mathematics and Statistics, University of Sheffield, Hicks Building, Hounsfield Road, Sheffield S3 7RH, UK

[2]UMR 5554 Institut des Sciences de l'Evolution, équipe Dynamique de la biodiversité, anthropo-écologie, Université de Montpellier, CNRS, IRD, EPHE Place Eugène Bataillon, 34095 Montpellier, CEDEX 05, France

[3]Department of Archaeology, University of Sheffield, Northgate House, West Street, Sheffield S1 4ET, UK

[4]Institute of Archaeology, 36 Beaumont St, Oxford, OX1 2PG, UK

Corresponding author: V.Bonhomme@sheffield.ac.uk

Abstract

The application of morphometric analysis in archaeobotany has the potential to refine quantitatively identifications of ancient plant material recovered from archaeological sites, most commonly preserved through charring due to exposure to heat. This paper uses geometric morphometrics, first, to explore variation in grain shape between three domesticated cereal species, einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*) and barley (*Hordeum vulgare*), both before and after experimental charring at 230 and 260°C. Results demonstrate that outline analysis reliably reflects known variations in grain shape between species and differences due to charring observed in previous experimental work, and is capable of distinguishing the species, with near-perfect results, both before and after charring. Having established this, the same method was applied to different accessions of the same species, which indicated that three different grain morphotypes of einkorn and two, possibly three, of emmer could be identified in the uncharred material, and that at least two different morphotypes for each species could be distinguished even after charring at temperatures up to 260°C. This opens up the possibility of tracking evolutionary change in crops, both chronologically and geographically, through morphometric analysis.

Highlights

- outline analysis reliably reflects known variations in grain shape between species, and differences due to charring
- more subtle differences in grain shape between different populations of the same species can be identified
- evolutionary change in crops could thus potentially be tracked, both chronologically and geographically, through morphometric analysis

Keywords: Elliptical Fourier Transforms, archaeobotany, cereal grain, experimental charring, einkorn, emmer, barley

1 Introduction

The taxonomic identification of ancient plant material found on archaeological sites is fundamental to its interpretation in terms of past plant use, agricultural practices etc., and these archaeobotanical remains are most commonly preserved through charring due to exposure to heat. Problems of taxonomic identification of charred plant remains are widely recognised (e.g. Jones, 1997; Hillman, 2000; Van der Veen, 1992). As well as natural overlap in morphology within and between species, distortion due to the charring process presents further difficulties for taxonomic identification. Cereal grains, which are largely composed of starch, are particularly susceptible to distortion through charring (Charles et al., 2015).

Although it is possible to identify well preserved cereal grains to species even after the distorting effects of charring, more subtle variations within species have not commonly been explored, due to the lack of reliable methods for distinguishing between different sub-species or varieties. Morphometric methods have been used to address intra-species variation for other archaeobotanical remains such as grape pips (Bouby et al., 2013; Terral et al., 2010), and Ros et al. have recently investigated grain shape variation between sub-species and varieties of barley (Ros et al., 2014). This type of investigation is best achieved through the analysis of variation in modern material where the species and source of the grain is already known, before attempts are made to apply the method to archaeologically preserved material where taxonomic identity must be inferred from the remains themselves. This paper explores the potential to refine quantitatively taxonomic identification though the geometric morphometric analysis of grain shape to determine the extent to which inter- and intra-species differences can be identified, both before and after charring.

Morphometrics, the description of shape and its (co)variation, encompasses three different approaches: “classic” identification, “traditional” morphometrics and geometric (also called “modern”) morphometrics (Bookstein, 1991; Rohlf and Bookstein, 1990). Archaeobotanical identification is classically based on a series of diagnostic traits, including descriptions of shapes, that are assessed by eye, and that can be recognised consistently by trained specialists. Identification by eye, however, leaves limited capacity for quantifying variation within or between archaeobotanical assemblages. In contrast, a morphometrics-based approach allows shape variation to be directly quantified and, further, plant remains can be classified probabilistically. The ability to quantify grain shape variation also holds great potential for tracing past phenotypic variation in cereal populations both temporally and spatially, thus documenting diversity, chronological change and geographic movements of cereal crops.

“Traditional” morphometrics, the measurement of linear dimensions (typically length, breadth and thickness for grains) and calculation of ratios of these dimensions, is occasionally used to aid identification of archaeobotanical remains, for instance between wild and domesticated varieties (Colledge, 2001). Measurements are not, however, routinely taken in archaeobotanical studies.

Geometric morphometrics represent shapes by quantitative variables using a mathematical framework defined by the nature of the shapes studied. The manner in which this is achieved depends on whether there are many features present that can be landmarked, or whether curves, outlines and surfaces are the shapes’ main homologous features. Recently, application of geometric morphometrics to archaeobotanical material has proven helpful to aid species identification (García-Granero et al., 2016) and, beyond this, to examine variation within species (Burger et al., 2011; Newton et al., 2006; Orrù et al., 2013; Pagnoux et al., 2014; Ros et al., 2014; Terral et al., 2012, 2010, 2004; Ucchesu et al., 2016). Studies to date have, however, focused on fruit stones such as grape and olive, while the application of geometric morphometrics to cereal grains has been treated with caution due to the known shape distortion caused by charring of starch-rich grain compared with the relative shape stability of woody fruit stones. As well as distortion depending on the type of material charred, the conditions under which charring occurred (e.g. temperature, oxygen availability, and, to a lesser extent, duration of heating) are also important (Bouby et al., *in press*; Charles et al., 2015; Ucchesu et al., 2016).

Previous work on cereals has demonstrated that grain distortion increases with charring temperature, with a noticeable difference between wheat grains charred at 230 and 260°C (Charles et al., 2015). Grains charred at these temperatures are comparable to well-preserved grains recovered from archaeological sites, both in terms of appearance and internal structure as seen through scanning electron microscopy (Charles et al., 2015). At higher temperatures, grain shape changes more dramatically, making it difficult to distinguish species and even genera; bubbles may appear on the grain surface and, in extreme cases, the endosperm is exuded from the grain (Braadbaart, 2008; Charles et al., 2015). As intra-species differences are unlikely to be preserved where species or genus is indeterminable, this paper focuses only on grains charred under conditions that generate well-preserved remains. Charring also causes an overall reduction in size but, as size is not a useful characteristic for distinguishing between grains of domesticated wheat and barley, we have restricted our analyses to shape differences.

For morphometric analysis of charred archaeological cereal grains to be considered meaningful, it must be established that, for well-preserved grains (charred at relatively low temperatures), the effects of charring do not obscure or distort grain shape to the point where variation due to charring is greater than the inherent differences between species or between different populations within species. The ability of morphometric analysis to distinguish between grains of known cereal species is also an essential pre-requisite for attempting to use the technique for exploring more subtle within-species variations. Having established this, an analysis of grain shape variation within species can follow.

Two key questions are therefore addressed: i) whether geometric morphometrics, specifically outline analysis using elliptical Fourier transforms, can satisfactorily distinguish modern grains of three domesticated cereal species commonly found archaeologically: einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*) and barley (*Hordeum vulgare*), in uncharred material and in material charred at 230 and 260°C; and ii) whether any of the accessions of grains from different populations of the same species exhibit characteristic shape differences and, if so, whether these are still distinguishable after charring. An ability to identify plant populations using geometric morphometrics, despite morphological changes due to charring, would indicate that this approach would be applicable to the archaeobotanical record and could then be used to seek out distinct cereal populations in antiquity.

2 Materials and Methods

2.1 Materials

Three accessions each of three cereal species, einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*) and barley (*Hordeum vulgare*), were included in this study. The accessions originated from various locations in Turkey, Jordan, Iran and Syria, and were provided by the John Innes Centre (UK), GRIN (USA) and IPK Gatersleben (Germany) (see supplementary material, Table A). At least 18 grains were sampled for each accession. For none of the accessions was the shape of the grains obviously distinctive, though grains of the einkorn accession Tm3 were unusually large compared to those of accessions Tm1 and Tm2. There was therefore no certainty, prior to analysis, that it would be possible to distinguish any of the accessions from others of the same species on the basis of their grain shape.

Grains were taken from spikelets throughout the ear, except for the very basal and terminal spikelets, where the grains are sometimes underdeveloped. Einkorn grains were taken only from one-grained spikelets and, for emmer, only two-grained spikelets were sampled (those containing one grain being, in any case, primarily from the bottom and terminal spikelets), and both grains in the spikelet were used. For barley, only two-row varieties were sampled. Wheat grains (einkorn and emmer) were dehusked by hand to remove the surrounding glumes. For barley grains (which were all of the hulled type), the paleas and lemmas were partially peeled off to expose the grain shape at the ends, and to better replicate archaeobotanical remains, where the complete hulls are rarely preserved.

Each grain was photographed in dorsal, lateral, and polar views, the latter capturing the cross-sectional shape of the grain (Figure 1; see also Jacomet, 2008) using a Leica Z6 apochromatic microscope, Retiga 2000R camera and Media Cybernetics® Image Pro Premier 9 software®.

2.2 Controlled charring

Each grain was given a unique identification code to facilitate one-to-one comparison of pre- and post-charring morphology. Individual grains were wrapped in two layers of aluminium foil and buried in sand within a 250ml Pyrex® beakers, thus reducing the availability of air during heating (Charles et al., 2015). Beakers containing half of the grains from each accession were placed in a pre-heated oven maintained at a temperature of 230°C (with an accuracy of ± 2°C) for 6 hours, and the other half were similarly heated at 260°C for 6 hours. After charring, each grain was photographed again in all three views, and with the same orientation as the uncharred grain.

2.3 Outline analysis

For each photograph, an outline of the grain was traced manually in Adobe® Photoshop® CS6 to capture the grain shape, excluding any protrusion of the embryo (see Figure 1). Two landmarks were defined in StereoMorph (Olsen and Westneat, 2015), one at the bottom of the image, at the embryo end of the grain or at the ventral groove depending on the view, and the other on the outline vertically above the first landmark (see Figure 1).

Outline coordinates and landmark positions on each image were then extracted and collated using Momocs 1.0.10 (Bonhomme et al., 2014 - https://cran.r-project.org/package=Momocs and https://github.com/vbonhomme/Momocs/) in an R 3.2.4 environment (R Development Core Team, 2016), where further analyses were conducted. Outlines were normalized before morphometric analysis as follows: for rotation and position, the two landmarks were superimposed on points with coordinates (x_1=-0.5, y_1=0), (x_2=0.5; y_2=0); for size, shapes were rescaled using their centroid size; for the bilateral symmetry of the grain, the polar view outlines were manually inspected and flipped so that the direction of asymmetry was the same in all grains.

Elliptical Fourier Transforms (Giardina and Kuhl, 1977; Kuhl and Giardina, 1982) were calculated for every grain in each view separately and later combined. Eight harmonics were retained for each view, which gathered at least 99% of the total harmonic power (Bonhomme et al., 2014). Eventually, each grain (both before and after charring) was described by 96 "Fourier coefficients": 3 views, described by 8 harmonics, with 4 coefficients per harmonic.

2.4 Statistical analyses

Mean coefficients per view, per species, and per charring state, were used to reconstruct mean shapes. To reduce dimensionality, a principal component analysis (PCA) was calculated on the matrix of coefficients. To visualize how the first principal component (PC) captures shape variability from the three views together, morphospaces were reconstructed at the origin and the PC1 and PC2 extrema.

To examine the effect of charring on shape, charring trajectories (Adams and Collyer, 2009; Collyer and Adams, 2013) are displayed on the PC1-PC2 plane. Each grain is represented twice, before and after charring. These points are linked by an arrow to define "charring vectors" or "trajectories" that display the change in shape on charring. Charring trajectories were decomposed into a direction and a magnitude, and non-parametric rank-based tests were used to compare between species (at a given temperature) and between charring temperatures (for each species). Kruskal-Wallis tests were used for three-group, and Wilcoxon tests for pairwise, comparisons.

To test whether outline analyses of grains can distinguish between species and between different accessions within each species, both before and after charring, linear discriminant analyses (LDA) with leave-one-out cross-validations were used. Each LDA used sufficient PCs to gather at least 95% of the total variance. For each within-species classification, the PCAs were recalculated on the matrix of Fourier coefficients filtered to include only grains of that species and charred at the considered temperature. The number of correctly reclassified grains, was used as a performance score.

3 Results

3.1 Changes in shape due to charring

Figure 2 shows the average change in grain shape due to charring in the three views photographed. It should be noted that size has been factored out of the analysis and so changes in the length and overall size of the grain are not apparent in these diagrams. In terms of shape, grains became generally rounder, with the most marked shape changes occurring in the polar/cross-sectional views of einkorn and emmer. Minimal changes in shape were observed for the lateral views of all taxa. In einkorn, there appears to be a reduction in, or even loss of, bilateral asymmetry in the polar view. Barley exhibited relatively little overall change in shape, which may be due to the incomplete removal of the hulls that could constrain deformation to some extent, or to the shallowness of the ventral groove in barley compared with the deep groove in wheat which tends to “open out” in the early stages of charring.

3.2 Exploration of shape variation in relation to species

Principal component analysis (PCA) of the Fourier coefficients calculated for grains of all species (uncharred grains and those charred at 230 and 260°C), based on all three views, shows (Figure 3) that, as expected, the greatest variation in grain shape is between species (PC1), and secondary variation is due to changes resulting from charring (PC2). Prior to charring, grains of the three species are clearly separated along PC1 with no overlap in the shape of grains of different species. Charring at 230°C shows some convergence of grain shape, but with little overlap between species, while charring at 260°C results in more overlap on the first two PCs for the two wheat species, though barley grains remain relatively distinct.

3.3 Magnitude and direction of shape changes caused by charring

The direction of charring vector trajectories (Figure 4) is broadly similar for most grains at both charring temperatures. The direction of change in shape is particularly similar for einkorn and emmer at 230°C but rather different for barley. At 260°C, the direction of change for einkorn remains similar whereas for emmer the direction alters slightly, becoming more like that for barley. There is a notable difference in the magnitude of the vectors, which is greatest for einkorn, intermediate for emmer and smallest for barley, reflecting the greater shape changes seen in the wheat species (cf. Figure 2). As expected, the magnitude of change is greater at 260°C than at 230°C. Kruskal-Wallis and Wilcoxon significance tests support these conclusions (see supplementary material, Table B).

3.4 Classification of grains according to species

Linear Discriminant Analysis (LDA), of the scores on sufficient PCs to account for ≥95% of the total variance in the matrix of Fourier coefficients, achieves perfect classification of all uncharred grains to species, even as assessed by rigorous leave-one-out cross-validation (rather than simple raw reclassification) (Figure 5a, Table 1). After charring at 230°C, a single grain of emmer was wrongly assigned as einkorn, while all other grains were correctly reclassified; the same results were observed after charring at 260°C (Table 1; and Figure 5b, displaying the combined results for grains charred at 230 and 260°C). This is a very encouraging result and demonstrates that outline analysis is capable of successfully discriminating between grains of different species, both before and after charring.

3.5 Discrimination between grains of different populations within species

When considering different accessions of the same species, it is not expected that every accession will have a distinctive grain morphotype compared to the other accessions of the species. The purpose of the analysis is to determine whether any of the accessions is distinctive and, if so, whether this distinctiveness can still be detected after the grains have been charred. LDA results for the uncharred grains of einkorn, however, show that all three accessions do exhibit a distinctive shape, as demonstrated by the near-perfect reclassification of grains, with only two grains of Tm2 being misclassified (Figure 5c, Table 1). For emmer, there is one very distinctive accession, Td3, but overlap in shape between Td1 and Td2, though these too are partly distinguishable, with 75% of Td1 grains, and 81% of Td2 grains, being correctly reclassified (Figure 5e, Table 1). Correct reclassification rates for the barley accessions, on the other hand, are low (Figure 5g, Table 1), indicating that they are all of a similar shape.

For the post-charring results, therefore, we focus on the accessions that were distinctive prior to charring (indicated in bold in Table 1; see also supplementary information, Table C). For einkorn, reclassification of Tm3 is perfect after charring at 230°C, and 72% of grains were correctly reclassified when grains charred at 230 and 260°C are treated together as “charred” (Figure 5d, Table 1). Tm1 and Tm2 are more difficult to distinguish after charring: at 230°C, 67% of Tm1 and 78% of Tm2 were assigned correctly but, when grains charred at the two temperatures are taken together, 42% and 61% respectively were correctly reclassified. Nevertheless, as a group, 86% of the grains from these two accessions were correctly reclassified to the group, indicating that two relatively distinct morphotypes of einkorn can be recognised after charring at temperatures between 230 and 260°C (Table 1). For emmer, Td3 also remained distinctive after charring, with 83% of grains being correctly reclassified after charring at 230°C, and 89% when grains charred at the two temperatures are taken together (Figure 5f, Table 1).

4 Discussion and Conclusions

As expected, modern uncharred grains of einkorn, emmer and barley are clearly distinguished by their shape. It is encouraging, however, that, bar one grain, they are also clearly identifiable, on the basis of outline shape alone, after charring at temperatures up to 260°C. The ability to identify species accurately using morphometrics both before and after charring is not particularly surprising, as a trained archaeobotanist can make these distinctions by eye.

This is an important result, however, first because the LDA reclassification rate is nearly perfect, regardless of charring temperature (230 or 260°C), which is particularly significant for archaeologically recovered grains where the charring temperature cannot be accurately determined. It is, however, possible, to make broad estimates of charring temperature on the basis of results from charring experiments, and grains charred at temperatures above 260°C “show gross distortion, becoming irregularly shaped, with severe surface blistering or crumpling of the endosperm, and occasionally endosperm exudations” (Charles et al., 2015). It would be wise, therefore, to exclude grains with these characteristics from investigations of intra-species shape variation, even if the grains can be identified to species on the basis of visible characteristics (with informal allowance for likely charring effects).

Secondly, the clear separation of species on the basis of outline analysis paves the way for the investigation of intra-species grain shape variation. Had it not been possible to distinguish species by this method, there would have been little point in attempting to use the same technique for identifying different populations of the same species because, where shape differences between grains of different populations exist at all, these are less pronounced. The accessions used here, for example, were not markedly distinctive, so it was uncertain whether any significant grain shape differences would be found even in the original (uncharred) material. In fact, LDA successfully distinguished three different morphotypes of einkorn and two, possibly three, of emmer. Not surprisingly the correct reclassification rates were lower in the charred material but at least two populations were clearly distinguished amongst the grains of both species after charring at temperatures up to 260°C.

The ability to quantify grain shape variation within species opens up the possibility of tracking grain morphotypes, not previously observable in archaeobotanical cereal assemblages, both temporally and spatially. Variations in cereal grain shape within species are likely to reflect genetic varietal differences or crop growing conditions, though the latter are perhaps more likely to be reflected in grain size than grain shape. This potentially allows us track evolutionary changes in crops through time, which may relate to selective pressures due to climate or human manipulation, as well as the geographic movement or spread of crop varieties owing to exchange or human population movements. It also permits an assessment of the degree of crop diversity at different times and geographic locations, as a means of identifying centres of genetic diversity indicative of rapid evolutionary change.

Evolutionary changes in crops and livestock may also be investigated through DNA analyses (Brown, 1999), and so both genetics and morphometrics have the potential to contribute to our understanding of the origins and spread of agriculture (Tresset and Vigne, 2011). However, both approaches face barriers when trying to investigate the domestication and evolution of crop species through the study of ancient material. Molecular approaches are often thwarted by the poor survival of DNA resulting from the effects of both charring and the age of the material, which has led to a focus on the genetic analysis of modern crop plants to determine the origins of crop species (e.g. Brown et al., 2009) and to investigate their subsequent spread (e.g. Jones et al., 2013, 2012). The destructive nature of ancient DNA analysis is also a disadvantage, which is not the case for shape analysis. For morphometrics, the changes in shape caused by charring are problematic: distinguishing inherent differences in grain shape from the confounding effects of charring (both the overall bias introduced by the charring process and the effects of variable charring conditions), in order to understand the evolutionary and ecological processes that took place, is a real challenge. In this paper, we have addressed both the ability of geometric morphometrics to identify differences in grain shape relating to known species and population differences, and to take account of the effects of charring through the analysis of modern cereal grains.

Our results have demonstrated first that geometric morphometric analysis, specifically outline analysis, reliably reflects known variations in grain shape between species, and differences due to charring observed in previous experimental work. Secondly, we have been able to identify more subtle differences in shape between different populations of the same species. This has opened up the exciting possibility of tracking evolutionary change in crops both chronologically and geographically.

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Figures

Figure 1: The three grain views photographed, with the outlines shown in red and the positions of landmarks indicated by black crosses.

Figure 2: Mean outline shapes of the grains. Grey shapes correspond to mean shapes of the uncharred grains; black outlines correspond to mean shapes of the charred grains. Note that grain size has been factored out of both the uncharred and charred outlines, to reveal only changes in shape.

Figure 3: Principal component analysis of the 96 Fourier coefficients calculated for grains of all species (before and after charring), based on all three views: a plot of PC1 against PC2, accounting for 68% of the total variance. Tm = einkorn, Td = emmer, Hv = barley. Convex hulls are displayed for each combination of species and charring treatment. In the background, a morphospace of the reconstructed shapes with the three views arranged as dorsal above, lateral left and polar right.

Figure 4: Charring vectors. Arrows represent, for each grain, the trajectory caused by charring on the PC1-PC2 plane of Figure 3.

Figure 5: Linear discriminant analyses of the scores on PCs accounting for ≥95% of the total variance in the matrix of Fourier coefficients: with species as the grouping variable (a) uncharred (b) charred; and with different accessions as the grouping variable for einkorn (c) uncharred - (d) charred, emmer (e) uncharred - (f) charred, and barley (g) uncharred - (h) charred. In each case, “charred” indicates grains charred at 230 or 260°C (both temperatures combined). The two linear discriminant axes are shown, along with convex hulls for the species, or accessions within species.

Tables

Table 1: Linear discriminant analysis. Percentages of grains correctly reclassified to species, or to different accessions within each species; for both uncharred grains, and grains charred at 230 or 260°C (both temperatures combined). Bold indicates accessions with the most distinctive grain shapes.

Supplementary material

Table A: Cereal accessions. Source and location information for the einkorn, emmer and barley accessions analysed.

Table B: Trajectory analyses. *P*-values from Wilcoxon and Kruskal-Wallis tests on magnitudes and directions of the charring vectors. Tm = einkorn, Td = emmer, Hv = barley.

Table C: Confusion matrices. Detailed results of the LDA reclassifications of species, and accessions within species (rows), for the uncharred grains, for grains charred at 230°C, charred at 260°C, and charred at 230 and 260°C combined (columns).

Table1

	Uncharred (%)	Charred (%)	N
Species			
Einkorn	100	100	55
Emmer	100	98	96
Barley	100	100	54
Einkorn accessions			
Tm1	**100**	**42**	19
Tm2	**89**	**61**	18
Tm3	**100**	**72**	18
Tm1+Tm2	**97**	**86**	37
Emmer accessions			
Td1	75	67	24
Td2	81	58	36
Td3	**100**	**89**	36
Barley accessions			
Hv1	72	56	18
Hv2	56	39	18
Hv3	72	56	18

Figure1

Click here to download high resolution image

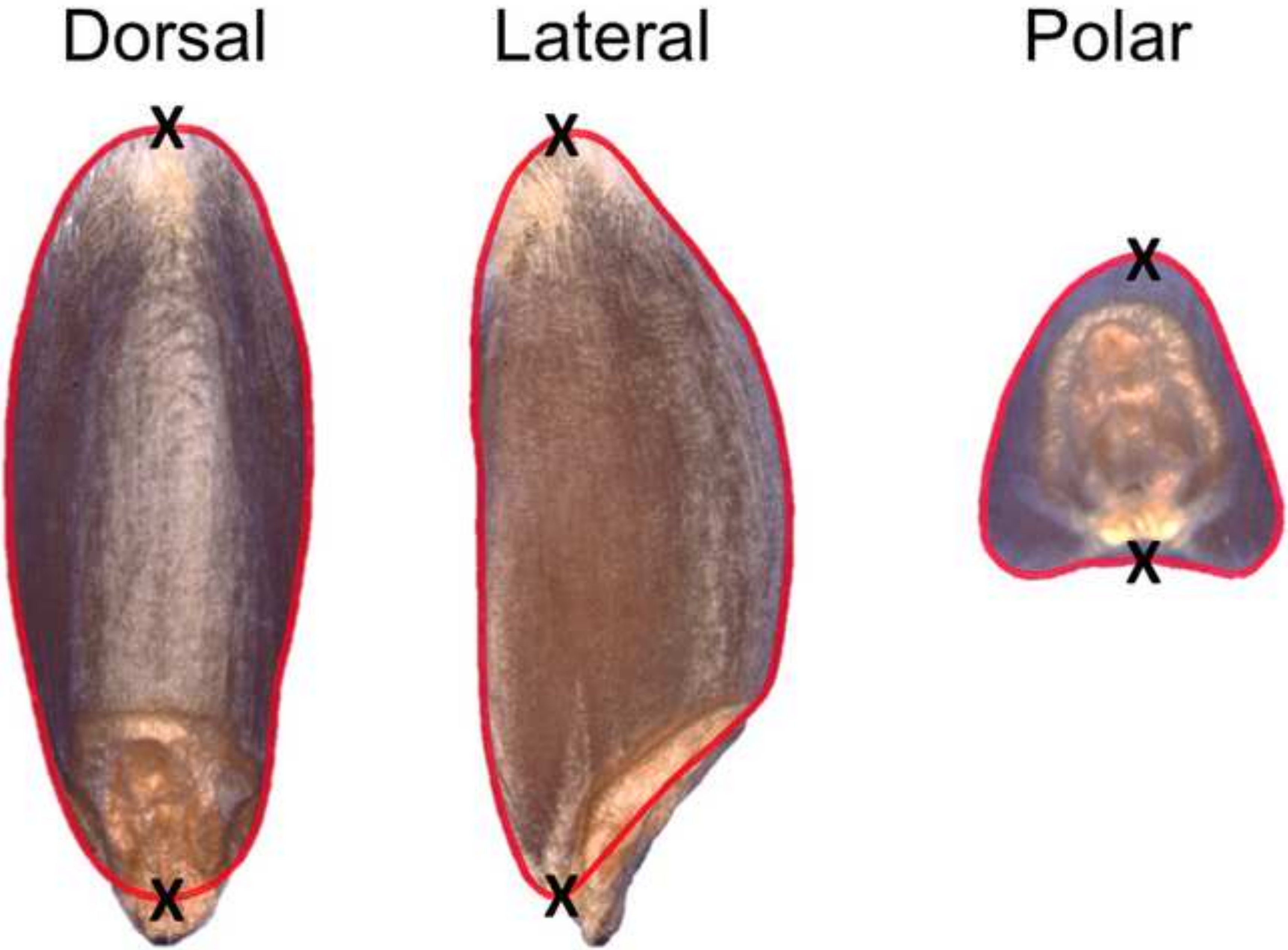
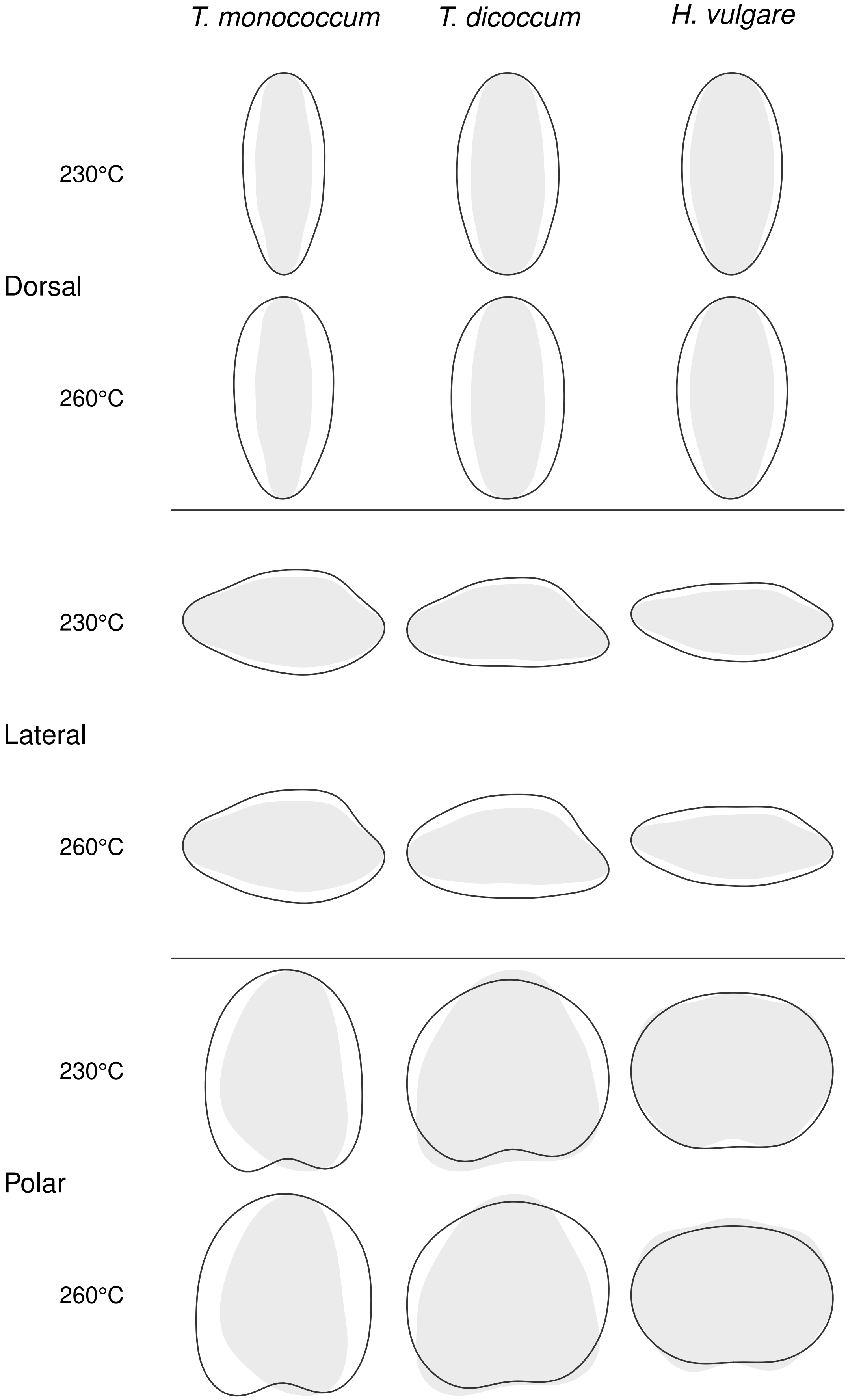


Figure2



T. monococcum
T. dicoccum
H. vulgare
230°C
Dorsal
260°C
230°C
Lateral
260°C
230°C
Polar
260°C

Figure3

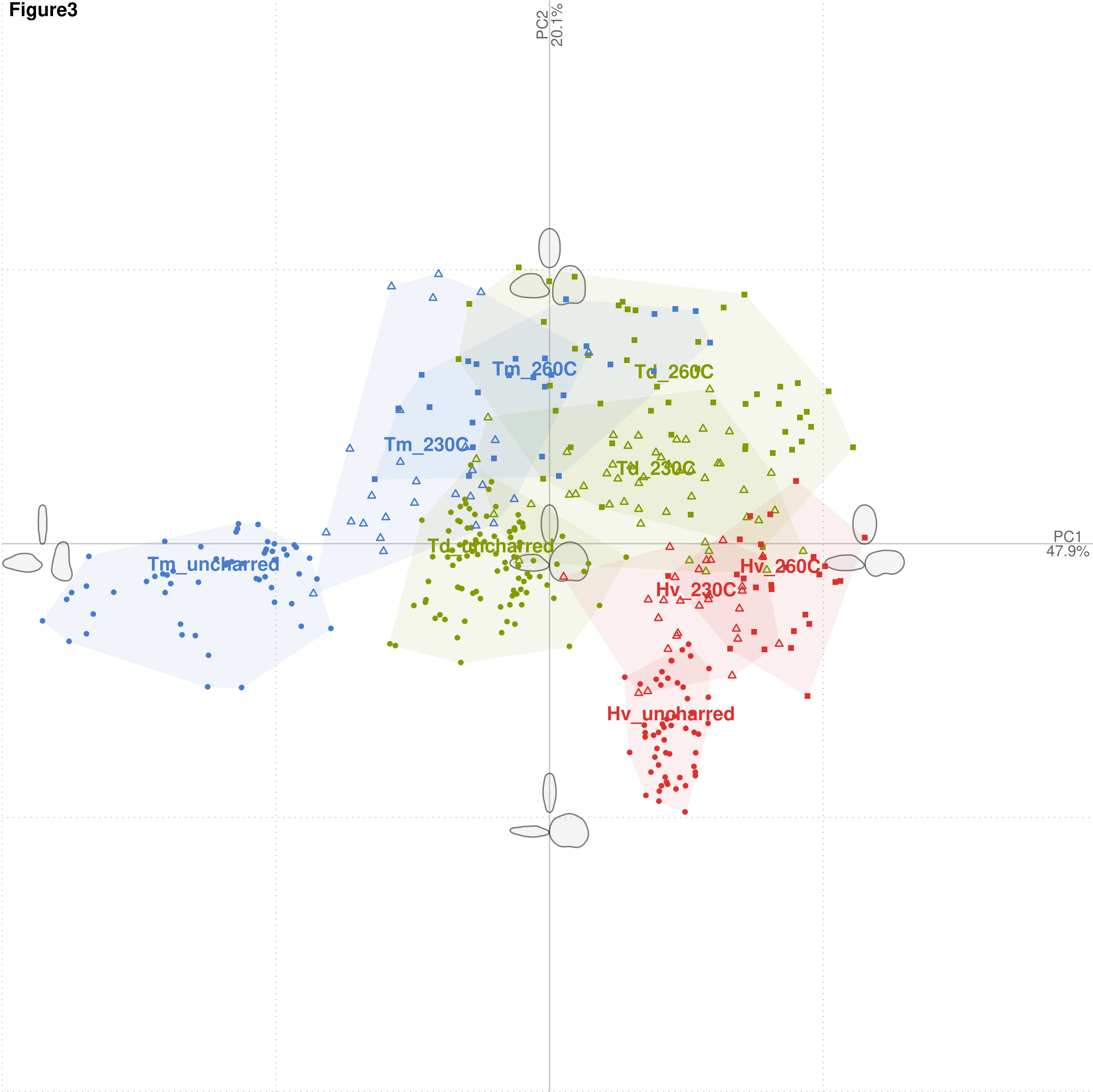

PC2
20.1%
PC1
47.9%
Tm_260C
Td_260C
Tm_230C
Td_230C
Td_uncharred
Tm_uncharred
Hv_260C
Hv_230C
Hv_uncharred

Figure4

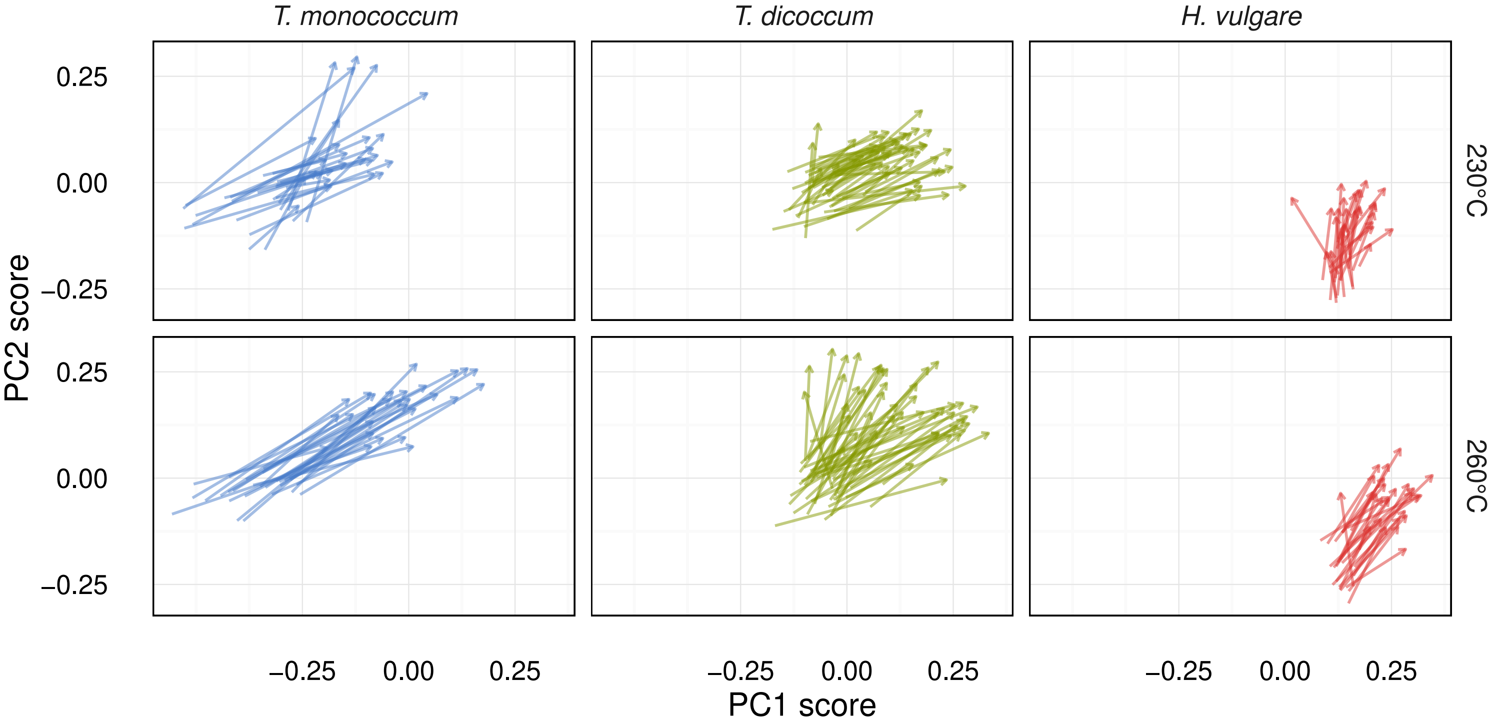
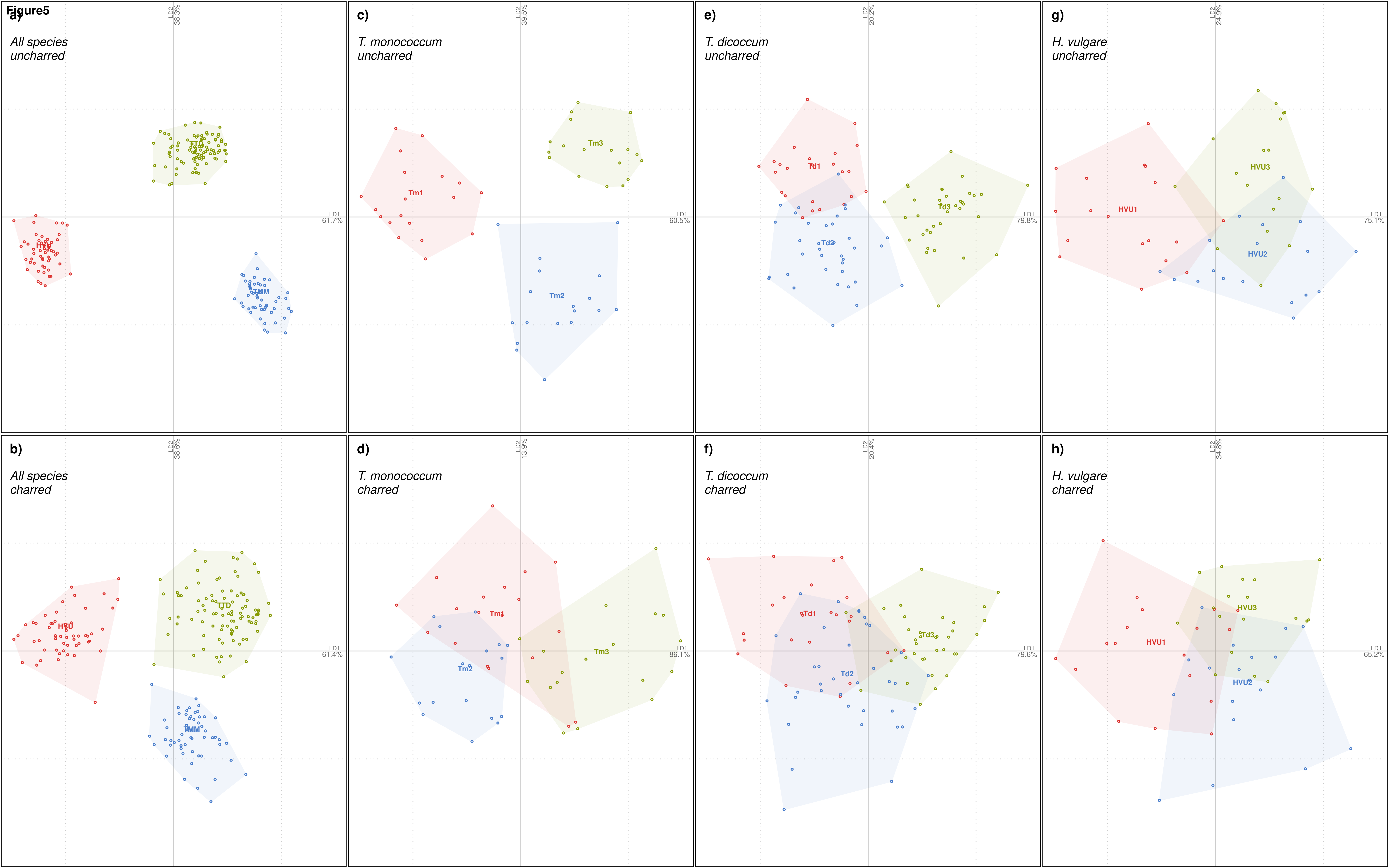

T. monococcum
T. dicoccum
H. vulgare
230°C
260°C
PC2 score
0.25
0.00
−0.25
0.25
0.00
−0.25
PC1 score
−0.25
0.00
0.25
−0.25
0.00
0.25
−0.25
0.00
0.25

Figure5


a)
All species uncharred
LD2 38.3%
LD1 61.7%
TTD
HVU
TMM
b)
All species charred
LD2 38.6%
LD1 61.4%
TTD
HVU
TMM
c)
T. monococcum uncharred
LD2 39.5%
LD1 60.5%
Tm1
Tm2
Tm3
d)
T. monococcum charred
LD2 13.9%
LD1 86.1%
Tm1
Tm2
Tm3
e)
T. dicoccum uncharred
LD2 20.2%
LD1 79.8%
Td1
Td2
Td3
f)
T. dicoccum charred
LD2 20.4%
LD1 79.6%
Td1
Td2
Td3
g)
H. vulgare uncharred
LD2 24.9%
LD1 75.1%
HVU1
HVU2
HVU3
h)
H. vulgare charred
LD2 34.8%
LD1 65.2%
HVU1
HVU2
HVU3

Supplementary Material - TableA

Click here to download Supplementary Material: TableA_CerealAccessions.pdf

Supplementary Material - TableB
Click here to download Supplementary Material: TableB_TrajectoryAnalyses.pdf

Supplementary Material - TableC
Click here to download Supplementary Material: TableC_ConfusionMatrices.pdf