

# Rare and neglected rice landraces as a source of fatty acids for undernourished infants

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**This study deals with the quantitative assessment of nutritionally important fatty acids (FAs) in 94 indigenous rice (*Oryza sativa* ssp. *indica*) landraces of India which are critically endangered, being cultivated only by a handful of marginal farmers. Three modern high-yielding varieties and one local high-yielding farmers' variety were analysed for comparative assessment. Qualitative and quantitative analyses of FAs based on gas chromatography-mass spectrometric analysis of these varieties revealed their significant contribution to daily diet. Two 'case studies' were considered on the basis of individual FAs and nutritionally correlated FA parameters of the rice cultivars to isolate the most promising landraces, by clustering through linear discriminant function. These folk rice landraces may add important precursors to essential FAs in the staple diet and can provide for FA requirement in normal brain development in infants. We suggest incorporation of these landraces into India's food and agriculture policy, both for conserving the vanishing landraces and for ensuring nutritional security of the economically marginalized people.**

**Keywords:** Fatty acids, gas chromatography-mass spectrometry, landraces, linear discriminant analysis, rice.

RICE is a major staple food crop of half of the world's population, grown in more than 115 countries, where it is the principal source of dietary energy<sup>1</sup>. India is one of the world's largest rice producers, covering 20% of the total global production, and contributing to daily caloric intake. Rice is known to contain various classes of fatty acids (FAs) as lipidic component, in addition to vitamins, minerals, starch and a small amount of protein<sup>2-5</sup>. There is growing consciousness on the importance of nutritional

richness, in addition to food availability and entitlement, for food security. The green revolution (GR) in the mid-1960s was geared to shape the Indian agricultural policy for combating hunger by the introduction of high-yielding varieties (HYVs), fertilizers, pesticides, irrigation facilities and free electricity<sup>6</sup>. The reliance of GR on industrial agrochemical 'input' has over succeeding decades resulted in accumulation of toxic contaminants in soil and water, gradual decimation of agrobiodiversity, dietary diversity loss and micronutrient malnutrition<sup>7-9</sup>. Concomitant with agricultural modernization, the traditional knowledge of agronomic characters, nutritional value of different landraces and different cooking methods to keep their nutritional properties, have faded away<sup>10</sup>. With an entrenched institutional apathy towards these landraces, the nutritional properties of the extant landraces or folk varieties (FVs) remain unexplored. The policy emphasis on grain yield increase in rice breeding programmes has engendered the paradox of a cumulative excess stock of rice<sup>11</sup> alongside widespread hunger and malnutrition in India, which currently ranks 94th out of 107 countries<sup>12</sup>. In particular, India has the most 'wasted' children under years 5 of age amongst the 107 countries assessed<sup>12</sup>.

Recent research has revealed that many forgotten rice landraces contain high levels of B complex vitamins<sup>13,14</sup>, metal micronutrients<sup>15,16</sup> and various ranges of antioxidant potential, phenols and flavonoids<sup>17,18</sup>, not reported in any modern HYV. This study extends the exploration of nutrients in the vanishing rice landraces to the regime of important long chain polyunsaturated fatty acids (LCPUFAs), to meet a significant part of the nutritional needs of the poor.

Since humans lack the substrate-specific desaturase enzymes, two essential FAs, namely alpha-linolenic acid (18:3  $\omega$ 3; ALA), and linoleic acid (18:2  $\omega$ 6; LA) obtained from plants or marine animals are the only dietary substances that are used in *de novo* synthesis of

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LCPUFAs in the human body<sup>19</sup>. ALA (18:3  $\omega$ 3) is the only plant-derived  $\omega$ 3 FA which reduces the production of lysophosphatidic acid, a factor for progression of neuropathic<sup>20</sup> and inflammatory pain<sup>21</sup>. Two other conditionally essential  $\omega$ 3 FAs are eicosapentanoic acid (20:5  $\omega$ 3; EPA) and docosahexaenoic acid (22:6  $\omega$ 3; DHA). EPA is the precursor of prostaglandin and plays a leading role in platelet aggregation<sup>21</sup>, while  $\omega$ 6 FAs and DHA are necessary to build the framework of the phospholipid component of the cell membrane and are responsible for maintaining membrane permeability, facilitating the transport of macromolecules and bioactive lipid mediator<sup>22</sup>. These polyunsaturated fatty acids (PUFAs) are also precursors of many secondary metabolites and play a significant role in signal transduction pathways and reducing reactive oxygen species (ROS)<sup>23</sup>. Mother's milk contains all these important growth-promoting factors, including EPA and other unsaturated fatty acids (UFAs) and LCPUFAs, in addition to a wide range of nutrients like proteins, carbohydrates and minerals. LCPUFAs like arachidonic acid (ARA, 20:4  $\omega$ 6), EPA and DHA can be biosynthesized from LA and ALA, and can also be obtained from dietary source<sup>24</sup>. Neonates and infants below 5 years of age have a very low level of elongase and desaturase activity for conversion of PUFA to LCPUFA<sup>25</sup>. Mother's dietary intake of PUFA has a direct correlation with PUFA composition of her milk, thereby ensuring the availability of PUFA in infants<sup>24,25</sup>. Dihomo- $\gamma$ -linolenic acid (C20:3  $\omega$ 6; DGLA) and ARA are formed by direct conversion of maternal dietary LA<sup>26,27</sup>. The concentration of these FAs in mother's milk along with other saturated fatty acids (SFA) and PUFAs is crucially determined by the mother's post-partum (30–360 d) dietary intake<sup>24</sup>. To meet the infant's metabolic need of 100 mg DHA/day, it is imperative for a breastfeeding mother to consume at least 200 mg of DHA per day<sup>27</sup>. We report here that certain rice landraces are an important source of these FAs.

## Materials and methods

### Rice samples

Samples of 94 FVs were procured from different geographical locations of the Indian subcontinent, conserved in the germplasm bank of Basudha Farm ([www.cintdis.org/basudha](http://www.cintdis.org/basudha)), located in Rayagada district, Odisha (19°33'06"N, 83°23'28.14"E), where all these landraces are cultivated *in situ* every year. The [Supplementary Table 1](#) provides origin of these landraces and morphological details. For comparison, seeds of three modern HYVs, namely Gitanjali (IET-17276), PNR 546 (IET-11347) and Sabita (IET-8970); and a local high-yielding landrace, Dudheswar, now grown in different parts of southern Bengal were procured from the Agricultural Experimental Farm of the University of Calcutta, at Baruipur (22°22'03.5"N and

88°26'07.2"E). All 98 landraces were subsequently grown in the 'kharif' season (June–December) in the above-mentioned farm. Each rice variety was grown on a separate plot at a density of 16 hills m<sup>-2</sup>. No synthetic agrochemical was applied in the farm, so as to eliminate the possibility of any chemically induced change in the metabolic pathway of rice plants.

### Analysis of fatty acid methyl ester

After harvest and drying, rice grains were decorticated with a small hand-made rice pounder (not by mechanical milling) and made into fine powder by crushing in liquid nitrogen. The total lipid was extracted from pulverized decorticated rice following the modified method of Bligh and Dyer<sup>28</sup>, modified<sup>29</sup>. FAs were trans-esterified into fatty acid methyl ester (FAME) using methanol/benzene/conc H<sub>2</sub>SO<sub>4</sub> (in the proportion of 4.3:0.5:0.6 by vol), kept at 85°C for 8 h, extracted with *n*-hexane (HPLC grade, E. Merck, India) and subjected to GCMS (Agilent Technologies, USA, 7890A GC system with 5975C triple axis detector MS attached with HP5-MS (30 m × 0.25 mm × 0.25  $\mu$ m) + 10 m Duraguard capillary column). The column temperature was programmed initially at 70°C with 1 min hold and ramping at the rate of 8°C/min up to 210°C with 5 min hold; subsequent ramping of 1°C/min up to 230°C and final ramping of 2°C/min up to 260°C. The flow rate of helium, as carrier gas was maintained at 1 ml/min. MS was conditioned with an ion trap at 200°C, transfer line temperature of 280°C at vacuum pressure of 2.21e–0.5 torr. Flame ionizing detector (FID) attached with GC (Varian, UK) was used keeping the same column condition. Analysis was performed using a capillary column (VF-1MS, WCOT, 15 m × 0.25 mm × 0.25  $\mu$ m Factor four<sup>TM</sup>). N<sub>2</sub> as carrier gas was maintained at a flow rate of 1 ml/min. Next, 1  $\mu$ l of sample was injected in both GCMS and GC-FID. Injection temperature and FID temperature were kept at 250°C and 260°C respectively. Identification of the peaks of FAME was done by calculating relative retention time (RRt), comparing with the authentic mixture of 37 FAME and PUFA (Supelco, Lot No: LB80556 and LB77207, USA) as standard, and by plotting log RRt against carbon chain length, and confirmed by scrutinizing mass fragmentation pattern of the compounds from NIST (2011) ChemStation software ([Supplementary Figure 1](#)). FAME was estimated by GC-FID. Each analysis was done in three replicates. The amount of FAs was calculated as relative percentage of total FAME.

### Statistical evaluation

The nutraceutical values of FAs of these rice grains were tested using two sets of parameters (case study I and II) using software R, version 3.4.2. The relative percentage

of all 27 FAs present in the rice grains of 98 varieties was estimated for statistical analysis and considered to be normally distributed for further processing.

In case study I, the relative percentage of 27 FAs present in 98 rice varieties was treated individually for statistical analysis. The variance was calculated by elbow method, explained as a function of the number of clusters. Based on this, five clusters can be obtained with 77.76% of variability. However, as the data pool in each cluster was too small, only three clusters were chosen explaining 65% of total variability. Subsequently, to segregate 98 rice varieties into these three clusters, non-hierarchical clustering, viz. 'k-means clustering method' was used, where the algorithm repeatedly reassigns cases (individual rice varieties) to clusters, so that the same 'case' moves from cluster to cluster during analysis for grouping of varieties in case study I. A 'case' was assigned to the cluster with the smallest distance so that the within-cluster variation was minimized. Each cluster thus formed was homogeneous, while being as different from the other clusters as possible. We subsequently conducted linear discriminant analysis (LDA) to allocate an individual variety to one of these three clusters on the basis of the 27 FAs and to assign the most 'likely' group. Using LDA, we projected the data onto a 2D plane so as to maximize the separation among the categories; while, preserving as much of the class discriminatory information as possible. In this process, only the analysed data of FAs of these 98 varieties of rice were taken into consideration for testing, but no new variety was used. From this we could determine the accuracy of the LDA function used by computing the apparent error rate (AER), calculated as the number of misclassifications per observation. The misclassification table was obtained with the AER of  $((3 + 5)/98 = 0.0816)$ . Therefore, the discriminant function has a small chance of misclassifying data, viz. 8.16% only.

For case study II, pooled FA parameters were selected for analysis of their nutritional significance. For example,  $\omega 6$  parameters were obtained by adding the relative percentage of all  $\omega 6$  FAs ( $\Sigma \omega 6$ ) as well as that of all  $\omega 3$  FAs ( $\Sigma \omega 3$ ) present in each rice variety. The ratio of  $\Sigma \omega 6 / \Sigma \omega 3$  was taken as a variable for calculation. Likewise,  $\Sigma ED = (EPA + DHA)$ , ARA,  $\Sigma UFA / \Sigma SFA$  ratio were computed. Thrombogenic index (TI) and atherogenic index (AI) were calculated on the basis of the above FA classes using the Ulbricht and Southgate<sup>30</sup> formulae, and were considered for statistical evaluation to assess the nutritional qualities of these 98 varieties. Overall, six nutritional parameters ( $\omega 6 / \omega 3$ , ED; ARA, UFA/SFA, TI and AI) were considered for clustering based on 77.9% variability among them, using the elbow method. The k-means algorithm was employed to assign the varieties in different clusters. LDA based on this clustering was performed to choose a discriminant function. Using this, we allocated each of the varieties into clusters and

determined the accuracy of the function by AER. While the three clusters were not distinguishable on a 2D plane, they were distinctly visible in the 3D scatterplot ([Supplementary Figure 2](#)). The AER was calculated as  $(2 + 1 + 1/98 = ) 0.0408$ . Therefore, the discriminant function has a small chance (4.08% only) of misclassifying data.

## Results

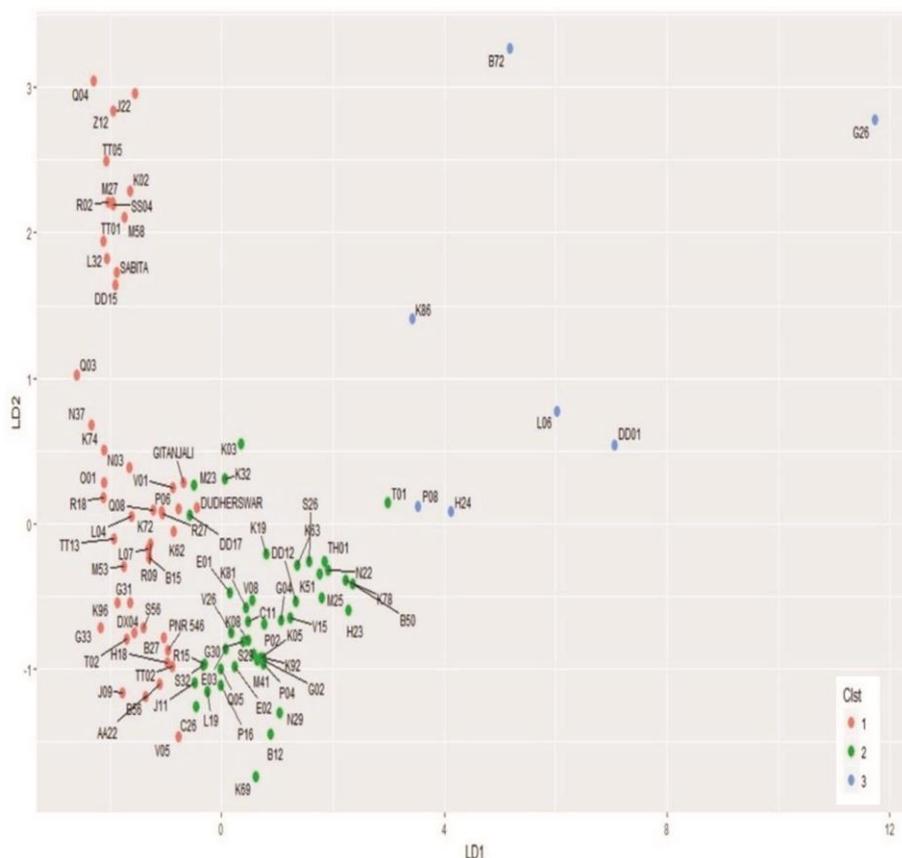
Rice contains various classes of SFAs and UFAs. Even carbon number FAs from C12 to C24 are common, while except for C15:0 and C17:0, FAs with odd carbon numbers are rare. Palmitic acid (C16:0) is predominant, ranging from 15.379% in K63 to 50.734% in J22 ([Supplementary Table 1](#)). Monoenoic fatty acids of carbon number 15 to 24 were also identified in most of these varieties, and 18:1n9c showed the highest abundance ranging from 5.259% in R02 to 56.998% in H23. Among PUFAs, C18:2 $\omega 6$ , C18:3 $\omega 6$ , C20:2 $\omega 6$ , C20:3 $\omega 6$ , C20:4 $\omega 6$  (ARA) and C22:2 $\omega 6$  were identified as  $\omega 6$  FAs and C18:4 $\omega 3$ , C20:5 $\omega 3$  (EPA), C20:4 $\omega 3$ , C20:3 $\omega 3$  and C22:6 $\omega 3$  (DHA) were identified as  $\omega 3$  FAs ([Supplementary Figure 1](#)). 18:2 $\omega 6$  (LA) ranged from 1.864% in J22 to 44.295% in K81, whereas 18:3 $\omega 3$  (ALA) was not detected in rice. ARA, one of the nutritionally important FAs varied from 0.284% in P06 to 31.739% in M27 ([Supplementary Table 1](#)). DHA (C22:6 $\omega 3$ ) found in 42 rice varieties ranging from trace to moderate (0.016% in DD17 to 2.251% in M27), whereas EPA (C20:5 $\omega 3$ ) was detected only in 15 samples in different amounts ranging from 0.002% in K81 to 1.393% in J22 ([Supplementary Table 1](#)).

Case study I reveals that FAs from C18:3n6 to C24:0 are the most effective in distinguishing cluster I from clusters II and III, whereas C14:0, C15:1 and C15:0 are indicator FAs for distinguishing clusters II and III. LDA obtained an overall 'good fit' and cluster I was segregated far away, although a reduction in dimension generated a few 'misclassifications' (in eight cases) for attributing vectors into clusters II and III. However, overall accuracy was more than 90% (AER = 0.0816).

Twenty-four rice varieties, namely DD15, E02, G33, J22, K02, K74, L07, L32, M27, M53, M58, N37, Q03, Q04, R02, R18, S32, Sabita, SS04, TT01, TT05, TT13, TT2 and Z12 segregated in cluster I (Figure 1). Cluster II has 32 varieties, including the modern cultivars Gitanjali and PNR 546, while cluster III consisted of 42 FVs (Figure 1). Using the LDA function based on clustering, the 24 rice varieties in cluster I were classified correctly. However, the LDA function misclassified five observations from clusters II to III, and three observations from clusters III to II.

In case study II, the nutritional parameter  $\omega 6 / \omega 3$  was the single most effective factor in determining the clusters. This ratio was significantly low in cluster I, considerably





**Figure 2.** Clustering of 98 rice varieties according to case study II. Numbers denote the variety codes according to the [Supplementary Table 1](#).

include a minimal amount of ARA in infant's milk, which is essential in the diet of 0–6 months infants in the range 0.2%–0.3%, and is still under clinical trial. However, a study on gene–diet interaction in the context of ‘Mendelian randomization’ has established the essential contribution of both  $\omega$ 3-DHA and  $\omega$ 6-ARA for cognitive development of infants<sup>27</sup>. Although cow's milk is often fortified and supplemented with iron, nucleotides, protein and LCPUFAs to prepare formula diet for infants, their pancreatic lipase is not mature enough to digest LCPUFAs present in these infant formula foods. In contrast, lipase from mother's milk increases the digestibility of FAs. In India, many FVs like Athikaraya, Dudh-sar, Kayamé, Neelam samba, Srihati, Maharaji and Bhejri are known in folk medicine to enhance milk production in lactating women<sup>34–36</sup>. Other FVs like Kelas, Dudhé bolta and Bhutmoori are rich in iron<sup>15</sup>, which can be included in the maternal diet to treat post-partum anaemia<sup>16</sup>. The present study reveals that 12 of 94 FVs examined could naturally supplement the nutritional demand of important FAs in undernourished mothers, and thus, may supplement the ARA and DHA in neonates through breast-feeding. This can be a more cost-effective and reliable measure than marketed formula foods.

However, rice intake does not contribute much towards ALA accumulation in the human body; so the major dietary uptake of ALA might be from other plant sources. Long-chain  $\omega$ 3 FAs, EPA and DHA can be synthesized from ALA, albeit with very low efficiency. In the human body,  $\omega$ 6 families of FAs are produced from C18 : 2  $\omega$ 6,  $\omega$ 3 from C18 : 3  $\omega$ 3,  $\omega$ 9 from C18 : 1  $\omega$ 9,  $\omega$ 7 from C16 : 1  $\omega$ 7 (ref. 37). The presence of dietary LA and ALA influences the ratio of  $\omega$ 6 and  $\omega$ 3 FAs, as these FAs are not interconvertible. Following the assertion by Simopoulos<sup>38,39</sup> that instead of the absolute values of  $\omega$ 6 and  $\omega$ 3, the  $\omega$ 6/ $\omega$ 3 ratio is a reliable health indicator, we have assessed this ratio as a variable in classifying the samples. An optimal ratio of 1 : 1 up to 4 : 1 of  $\omega$ 6/ $\omega$ 3 in the diet has been recommended to address the challenge for obesity management and risks of cardiovascular diseases and cancer<sup>38</sup>.

Using two independent statistical case studies, we have identified 12 nutritionally superior FVs ((DD15, J22, K02, L32, M27, M58, Q04, R02, SS04, TT01, TT05 and Z12) and an improved semi-tall variety (Sabita)) to be decidedly better for physiologically important FAs for human health (Figures 1 and 2). These 13 FVs have origins in different states of India, viz. seven from West

Bengal, two from Karnataka, two from Odisha, one from Jharkhand and one from Maharashtra; and one from Bangladesh as well ([Supplementary Table 1](#)). The other two modern cultivars (Gitanjali and PNR 546) and the local landrace Dudeshwar were also segregated in cluster I of case study II, but with less potentiality (Figure 2).

## Conclusion

The GR eliminated a wide range of native crops and their locally adapted landraces, characterized by superior grain yields than the HYVs in marginal farm conditions<sup>40,41</sup>. The reliance of GR on toxic agrochemicals and monoculture of HYVs has not only undermined food security of the poor and marginal farmers<sup>42</sup>, but also nutritional security of the consumers, as a multitude of landraces rich in nutrients have been replaced with nutrient-poor HYVs<sup>9,16,41</sup>. The present findings reveal that many FVs have significantly high levels of ARA, ED and low  $\omega 6/\omega 3$  values; yet they are on the verge of extinction from farm fields. *In situ* conservation of these neglected and vanishing landraces, considerably rich in crucial nutrients, is a cheaper and more rational option than industrial fortification of nutritionally inferior HYVs, to address the deepening problem of abysmal undernutrition in under five children in India, which ranks among the top 10 of the world<sup>12</sup>. It is imperative to design a constructive road map for regional nutrition security by cultivating the nutritionally rich folk rice landraces in marginal soil and climatic conditions.

**Conflict of interest:** The authors declare no conflict of interest.

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