cal, some of Kharia tribe's forefathers either brought wives from West Bengal or from North East region and settled here.

Further molecular studies are needed on the polymorphism and haplotype studies of haemoglobin variants, especially among the tribals of Orissa.

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Occurrence of a *Hop stunt viroid* (HSVd) variant in yellow corky vein disease of citrus in India

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A viroid was isolated and purified from total nucleic acid extract of *Kagzi* lime (*Citrus aurantifolia*) leaves affected by yellow corky vein disease. It was cloned in pGEMT-easy vector system and sequenced. *In silico* analysis showed that it consisted of 295 nucleotides. In BLAST analysis the sequence aligned with different *Hop stunt viroid* (HSVd) variants showing nearly 100% sequence identity with six citrus cachexia isolates of HSVd. The viroid was tentatively named as yellow corky vein variant of *Hop stunt viroid* (HSVd-ycv). This constitutes the first report of molecular evidence for occurrence of a *Hop stunt viroid* variant from citrus in India.

VIROIDS are low molecular weight, infectious, non-encapsidated, self-replicating, circular, single-stranded RNA molecules (246–463 nt) without any functional ORFs in their genome[1]. Ever since the first report of viroid[2] in potato, 32 viroid species[3] and 160 viroid variants[4] with complete sequence data have been recorded. These rap-

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idly evolving pathogens cause diverse diseases on a large number of economically important fruit and ornamental crops[5]. In India, tomato bunchy top disease was shown to be caused by a viroid[6]. Since then, association of a viroid-like RNA has been established with a number of diseases. We report here the molecular evidence for the association of a *Hop stunt viroid* variant with yellow corky vein disease of citrus (CYCVD). About three decades ago, Reddy and coworkers[7] reported this disease from Andhra Pradesh in sweet orange (*Citrus sinensis* cv. *Sathgudi*) showing yellowing and corky appearance of the veins. Later it was reported from Maharashtra[8]. The disease remained insignificant until *Kagzi* lime orchards were found to be affected by yellow corky vein disease, leading to 51.3–60.4% yield loss with an average fruit weight loss of approximately 90%, from Assam[9]. Etiological studies revealed that, though the causal agent was sap and graft transmissible, it was not a virus, but a low molecular weight RNA similar in electrophoretic mobility to viroid RNA[10].

The low molecular weight RNA was infectious and reacted positively to cRNA probes to *Citrus exocortis* and *Hop stunt viroids* in dot blot hybridization indicating that yellow corky vein disease may be caused by a mixed infection of at least two viroids[11]. But neither of them was separately isolated, purified or characterized. Therefore, the present investigation was undertaken for the isolation and molecular characterization of one of the components, the *Hop stunt viroid* variant.

Total nucleic acids were extracted from symptomatic leaves of glasshouse maintained *Kagzi* lime plants by the direct phenol method and low molecular weight RNA was purified by 2 M LiCl fractionation[12]. The low molecular weight RNA band was resolved in return PAGE (R-PAGE) as described by Singh and Boucher[13]. The RNA isolated from infected leaves of *Kagzi* lime was used as template for cDNA synthesis using Qiagen omniscript RT kit. For the first strand cDNA synthesis, 200 pmoles/µl of reverse primers of the two sets (Table 1) along with other reagents was added to the tube. The temperature profile and cycles performed were: 1 cycle for denaturation of DNA at 94°C for 2 min, 40 cycles which had three segments: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, chain extension at 72°C for 30 s. The double-stranded cDNA was then cloned in pGEMT easy vector system I (Promega)[14]. Recombinant plasmids were grown in *Escherichia coli* strain DH5α for further analysis. Clones were confirmed by colony PCR. Selected cDNA clones were sequenced by dideoxy chain termination method[15]. Sequences were searched in BLAST (http://www.ncbi.nlm.nih.gov/blast/) and compared with the sequences of other viroids obtained from GenBank database. The most stable secondary structure of the RNA was determined by the computer program RNA DRAW (version 1.1)[16]. To assess possible relationships with other viroids phylogenetic relationships were estimated by the CLUSTAL X program (Version 1.81b)[17].

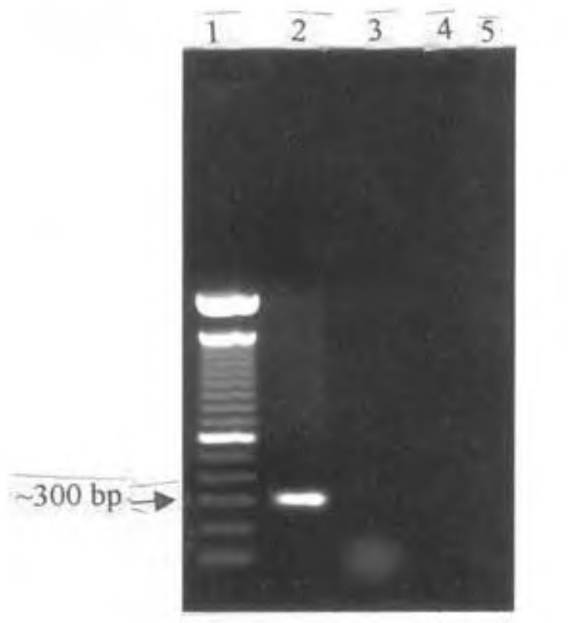

Figure 1. 1% Agarose gel electrophoresis of RT–PCR amplified product. Lane 1: 1 kb DNA ladder; Lane 2: Amplicon of ~300 bp size with RFL CVII(F)/RFL CVII(R) primer set; Lane 3: No product with RFL 44(R)/RFL 54(F) primer set; Lane 4: Reagent control; Lane 4: Healthy control.

Electrophoresis of total nucleic acid extracts from symptomatic leaves of CYCVD in R-PAGE revealed the presence of a low molecular weight RNA band, similar in electrophoretic mobility to nucleic acid from an isolate of potato spindle tuber viroid maintained in our glasshouse. The viroid RNA under study amplified in RT–PCR with only one set of primer RFL CVII(R)/RFL CVII(F), gave an amplicon (~300 bp) of expected size (Figure 1). Recombinants generated after cloning the amplified product were identified by colony PCR and selected clones sequenced.

Sequence analysis revealed that the viroid consists of 295 nucleotides (accession no. AJ490824), composed of 58 A, 87 C, 82 G and 68 U residues, thus resulting in a high G + C content of 57.29% and a high G + C/A + U ratio of 1.34, suggestive of a highly base paired, heat stable molecule, characteristic of viroid-like low molecular weight RNA.

The most thermodynamically favourable (at 37°C the energy is –85.61 kcal) secondary structure of the viroid RNA generated by RNA DRAW (version 1.1) software, consists of a highly base paired, quasi double-stranded rod, in which short helical regions alternate with internal and bulge loops (Figure 2).

In BLAST analysis the sequence aligned with different *Hop stunt viroid* (HSVd) variants showing nearly 100% sequence identity with six citrus cachexia isolates of HSVd [accession nos. AF131250, AF213491, AF213492, AF213493, AF213494, AF213495], but it has compara-

Table 1. HSVd primers used for PCR amplification

Primer	Sequence	Length (nt)	Expected size (bp)
RFL 44 (R)	5′CGC GGC AGA GGC TCA GAT AG3′	20	~300
RFL 54 (F)	5′GAT CCT CTC TTG AGC CCC TC3′	20	~300
RFLCVII (R)	5′GTC GCG TCT CAT CGG AAG AGC3′	21	~300
RFLCVII (F)	5′CGG TGG CAT CAC CTC TCG G3′	19	~300

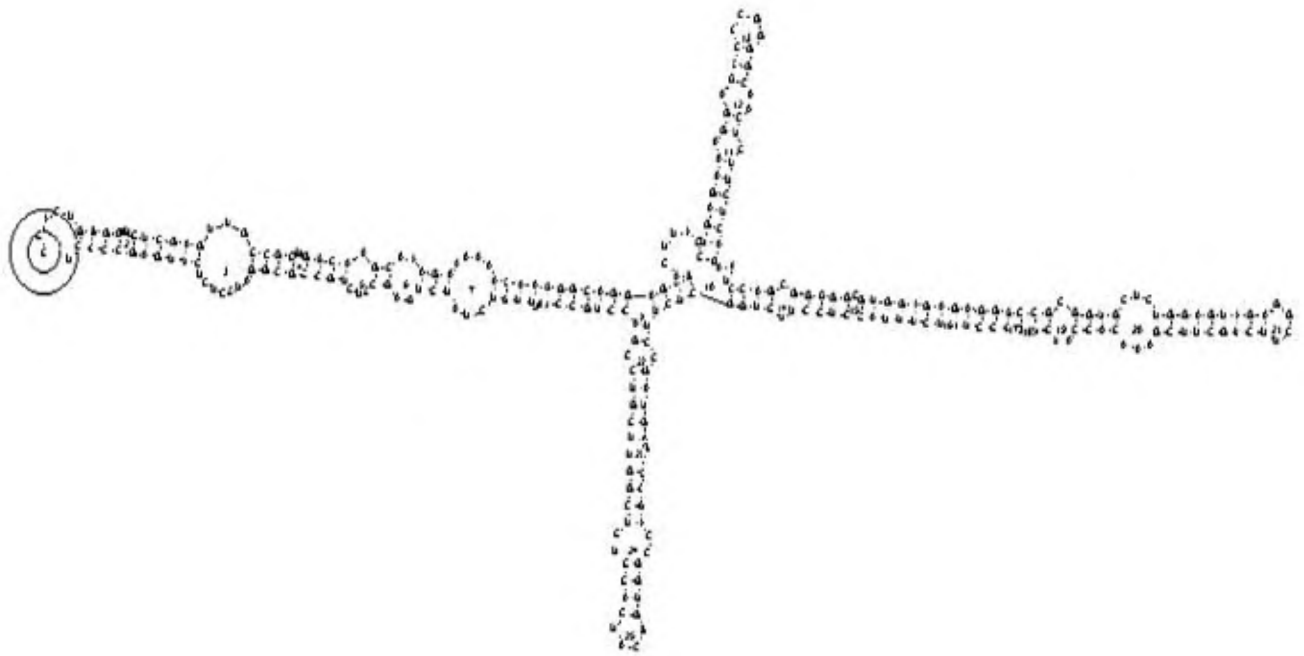

Figure 2. The highly base paired most thermodynamically favourable secondary structure of viroid under study predicted by RNA DRAW (version 1.1) software.

tively low homology with grapevine, cucumber, prunus plum and almond isolates of HSVd.

Based on this alignment result, it is apparent that the viroid under study belongs to the citrus isolate (or cachexia isolate) of HSVd and thus named as yellow corky vein variant of HSVd (HSVd–ycv), with its taxonomic placement in Hostuviroid group under the subfamily Pospiviroinae and family Pospiviroidae[18].

The 88 HSVd variants known till date have been placed in seven groups based on host reactions, viz. citrus isolate or cachexia isolate (HSVd.cit., 45 variants), grapevine isolate (HSVd.g., 9 variants), hop isolate (HSVd.h., 3 variants), prunus isolate (HSVd.apr., 24 variants), plum and peach isolate (HSVd.p., 3 variants), cucumber isolate (HSVd.c., 2 variants) and almond isolate (HSVd.alm., 2 variants). Total 30 sequence variants representing these seven groups were selected from http://callisto.si.usherb.ca/~jpperra/ and phylogenetic analysis of the test variant under study (HSVd–ycv) carried out with the selected variants using CLUSTAL X software to understand their evolutionary relationship (Figure 3). It was observed that the variants under the citrus isolate appear to be divided into three clusters. The HSVd–ycv belongs to the cluster where six variants are present; all of them are reported to cause Xyloporosis or cachexia disease of citrus. Among these, the severe variant Ca905 or CVd-IIc or HSVd-cit. 9 (AF131250) was reported on *Citrus medica* from California[19] but the other five (HSVd-cit. 20–24, AF213491–AF213495) were reported from Spain, which indicated that there is not much variation within the Old World and New World viroids. Most of the mild variants of citrus isolate are present in other clusters. It was also seen that HSVd–ycv has a distant relationship with variants belonging to other HSVd groups mentioned earlier.

In citrus a continuum of viroid-like RNA in the range of 275–375 nt was reported that varied in their infectivity on a series of citrus indicator hosts[5]. Based on molecular weight, hybridization and specific characters, these are grouped into five groups, viz. *Citrus exocortis viroid* (CEVd), *Citrus viroid* group I (CV I), *Citrus viroid* group II (CV II), *Citrus viroid* group III (CV III), *Citrus viroid* group IV (CV IV), each with subgroups[5]. Although five groups of viroid were recognized to be associated with citrus, only two groups, viz. CEVd and CV II subgroup b

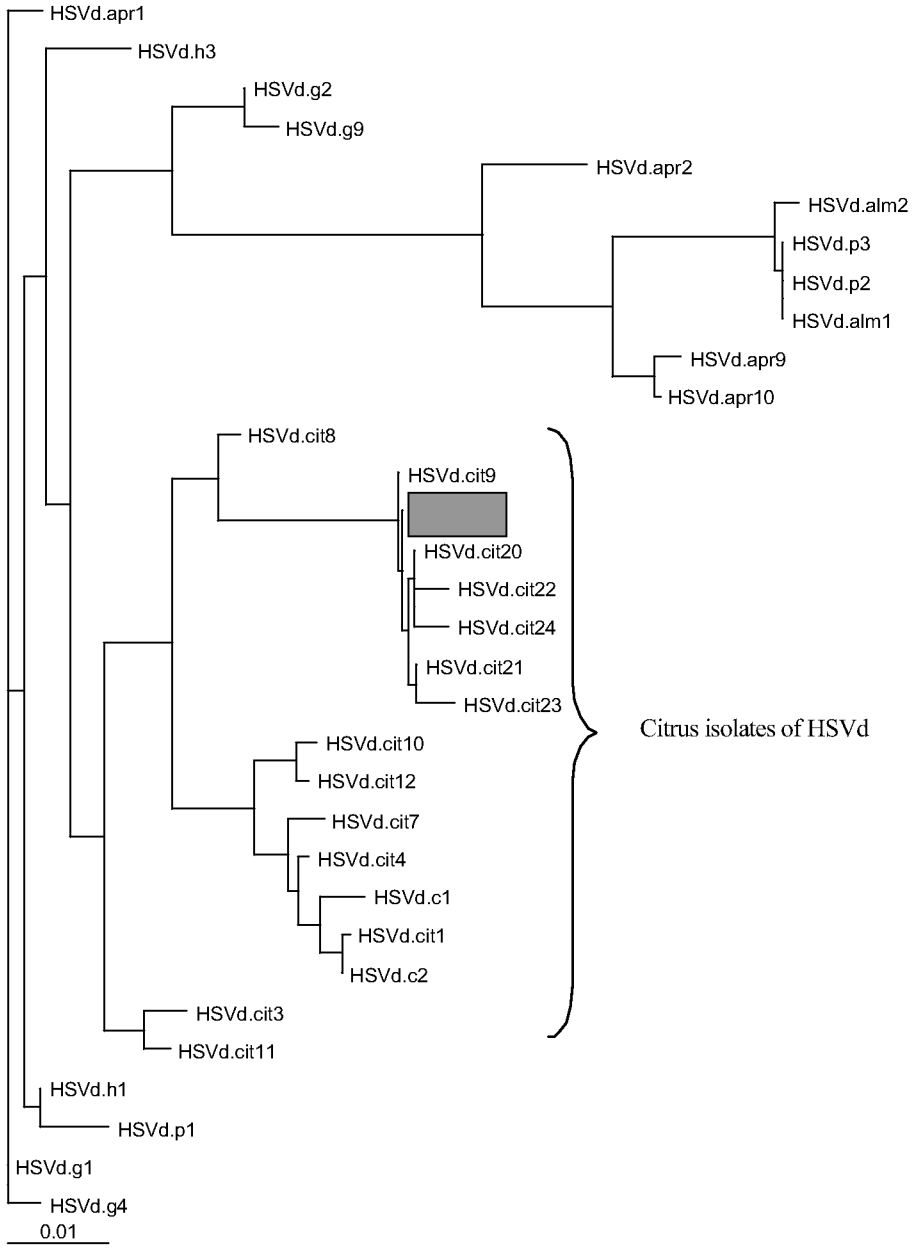

Figure 3. Phylogram, drawn by Neighbourhood Joining Bootstrap Method in CLUSTAL X (1.81b) software, illustrating phylogenetic relationships based on multiple alignments of the complete genome sequence of different hop stunt viroid variants and the yellow corky vein variant of hop stunt viroid (HSVd–ycv) under study (shaded block). Sequences and designation of other viroid variants for comparisons were obtained from http://callisto.si.usherb.ca/~jpperra/.

were shown to cause apparent disease in citrus, exocortis and cachexia respectively. However, the increasing number of sequence variants of a viroid appears to be a cause of concern in terms of their pathogenic ability.

Yellow corky vein disease of citrus is reported only from India[11]. Grafting transmits the disease and at least two viroids are associated with the disease. Between these two, one viroid component has been characterized during the present investigation. Results of R-PAGE analysis of total nucleic acid extract from yellow corky vein diseased *Kagzi* lime leaf confirmed the previous report that suggested association of viroid with the disease[10]. Rustem and coworkers[11] indicated that the disease might be caused by a mixed infection of two viroids of which one may be similar to HSVd. The PCR amplification with HSVd-specific primers produced an amplicon of ~300 bp size, similar to that of HSVd confirming the above observation. Sequencing of the cloned component revealed that

this viroid is a new variant of HSVd and was tentatively given the name yellow corky vein strain of HSVd (HSVd–ycv, accession no. AJ490824). The present investigation constitutes the first record of detection of a HSVd variant in citrus in India and also molecular characterization of a viroid infecting citrus. This HSVd variant, named HSVd-ycv, is a new viroid variant, which merits investigation in terms of its pathogenic ability to other hosts.

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Prediction of seed longevity in the genebank: How reliable are the estimates?

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Germplasms of crop plants are stored as seeds in genebanks at low temperature with low seed moisture content where they remain viable for several decades. The longevity of seeds stored in genebanks is predicted using viability equations developed by subjecting seeds to accelerated ageing under controlled laboratory conditions. We discuss here the serious consequences of provisionally or unreliably developed estimates of seed longevity parameters in making such predictions. A slight under or over estimation to the tune of 0.01 of the linear temperature parameter may result in a difference ranging from 46 to 74 years in the expected longevity. Whereas in case of the quadratic temperature parameter, a minor estimation difference (0.0001) may cause a difference of 11 to 12 years. A nonlinear estimation method based on Levenberg–Marquardt iterative convergence algorithm was applied for the reliable estimation of viability parameters for *Lupinus polyphyllus* seeds. The said estimation procedure resulted into comparatively narrow confidence intervals; and almost four to five times gain in precision over the conventional linear estimation in estimating potential longevity and moisture sensitivity parameters.

SEEDS are stored in the genebank under low moisture and temperature conditions to enhance their longevity. Prediction of storability of samples is essential to plan periodic regeneration and replacement. Seed longevity is mainly influenced by the environmental conditions such as storage temperature and moisture content of the seeds. Since seeds remain viable for several decades under practical storage conditions, conducting real time experiments to know storability of seeds are not feasible. Instead, seed longevity is determined under accelerated ageing conditions (i.e. high temperature and high moisture) and these results are extrapolated to predict longevity under genebank storage conditions. During the last three decades several attempts have been made to quantify the relationship between seed longevity and storage environment. Such relationships have been described by the viability/

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