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research paper

Haplotype variation and phylogenetic analyses of three commercially important morphological variants of genus *Turbinella* from the southeast coast of India based on the 16S rRNA gene

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ABSTRACT.—Haplotype variation and phylogenetics of three economically important morphological variants of *Turbinella pyrum* (Linnaeus, 1767)—var. *obtusa*, var. *acuta,* and var. *globosa*—were elucidated based on molecular analysis of mitochondrial 16S rDNA signatures. Haplotype distribution indicated that the var. *obtusa* was unique as revealed by the absence of any shared haplotype with the other two variants. Diversity indices depicted overall high mtDNA diversity for the morphological variants. Analysis of molecular variance revealed distinct population structure in the three studied variants. Based on haplotype diversity (H) and genetic distances, highest variation was observed between var. *obtusa* and the other two variants, while lesser variation was observed between var. *acuta* and var. *globosa*. However, highest nucleotide diversity (π) was observed between the var. *acuta* and the other two variants. Phylogenetic analyses also distinguished a phylogroup of var. *obtusa* highlighting its uniqueness. Overall, findings of the present molecular-based analysis indicated substantial genetic variation among the studied morphological variants of genus *Turbinella* and revealed that the morph *obtusa* was quite distinct from *acuta* and *globosa*.

The sacred chanks, *Turbinella pyrum* (Linnaeus, 1767) and *Turbinella rapa* Lamarck, 1816, are two of the most ecologically and commercially important gastropods in India. They dominate the shell craft cottage industries of India and are commonly used for making fine handicrafts and ethnic jewelry (Mukundan 1968). For a majority of the artisanal fishers inhabiting the coastal belts of Tamil Nadu, Gujarat, and the Andaman and Nicobar Islands, shell trade of the genus *Turbinella* is key to their livelihood (Venkataraman et al. 2012). Moreover, the shell ash and flesh of these gastropods are believed to have medicinal properties and have been used in indigenous medicinal practices for treating several diseases (Iyer 1933, Nadkarni and Nadkarni 1976, Sharma 1987, Radhika et al. 2008). The species within the genus

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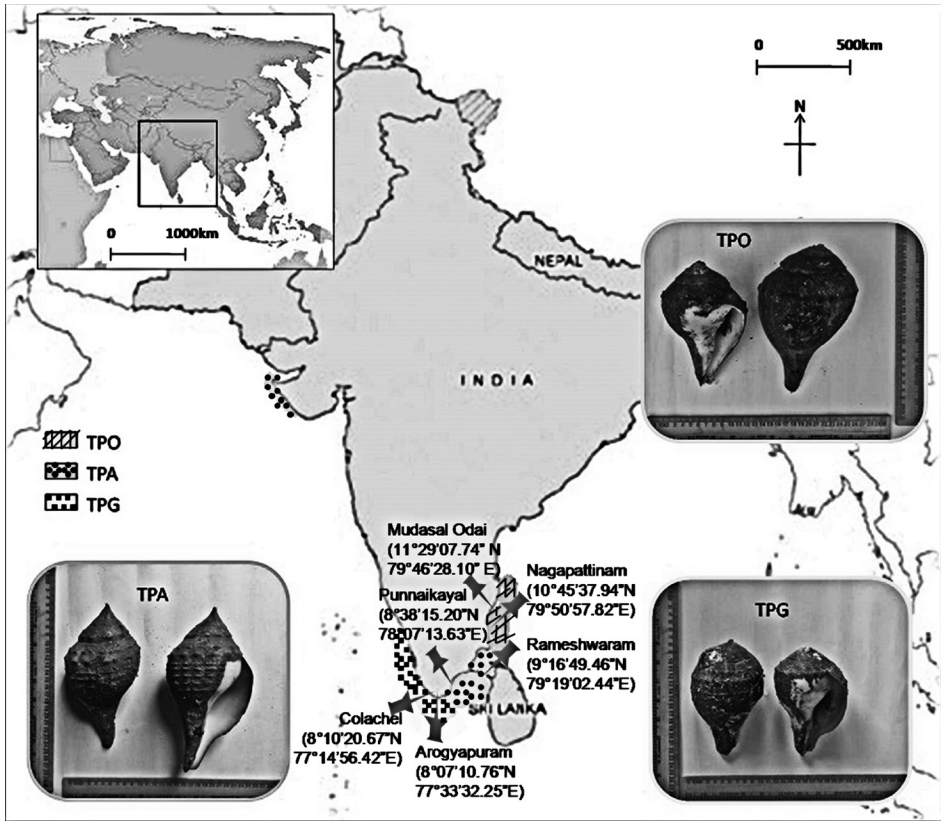

Figure 1. Distribution of three morphological variants of genus *Turbinella* off India: var. *obtusa* (TPO), var. *acuta* (TPA), and var. *globosa* (TPG).

Turbinella are confined to Indian waters with the exception of the adjacent coasts of Sri Lanka (Hornell 1949, Jones 1966, Nayar and Mahadevan 1973); especially high abundance of these chanks occurs on the east coast of India, particularly from Cape Comorin to the city of Madras (Gaur et al. 2005).

Turbinella pyrum and *T. rapa* possess massive, thick shells and have separate sexes. One of the major characteristics of this genus is the presence of three strong columellar ridges on the columella. However, the trace of a fourth ridge is typical to those residing in few regions of the distribution (Fig. 1). Twenty years is estimated to be the maximum life span of these chanks (Jagadis et al. 2010). January to March is reported to be the main breeding season for *Turbinella* and its larvae are reported to mature via direct development (Bandel 1975a,b). Typically, it is found at depths from 10 to 27 m and is rarely observed in shallow waters. With the principal food resource of polychaete worms, sandy bottoms with a moderate proportion of mud is their preferred habitat (Venmathi Maran 2001).

In 1916, Hornell (1916) proposed five varieties of *T. pyrum,* namely var. *obtusa*, var. *acuta*, var. *fusus*, var. *globosa,* and var. *comorinensis* based on their zoogeographic and eco-phenotypic attributes. These five varieties have their particular geographical distribution (Fig. 1) and characteristic physical and biological environment (Hornell 1915). The former three varieties are considered to be equal in importance commercially; among which the var. *fusus* is a very well-marked variety of *T. pyrum*, but its

occurrence is very rare on the Indian mainland coast with Andaman waters being their native breeding grounds. The latter two varieties overlap geographically and are known to be bathymetric variants of a geographically distinct entity (http://www.jaxshells.org/pyx.htm). In 1939, Winckworth (1939) classified *T. pyrum* var. *acuta* as a separate species, i.e., *Turbinella rapa* Lamarck, 1816 (Sartori 2015), based on conchology (Winckworth 1939). However, Hornell's (1915) classification of *T. pyrum* varieties is still adopted in the fishery and shell trading. Recently, a study examining shell morphology and adult specimen weight, including microsatellite-based characterization of var. *obtusa*, var. *acuta,* and var. *globosa,* revealed significant differences for six out of seven studied morphological characteristics (Arularasan et al. 2016). Microsatellite analysis also revealed significant genetic variation, wherein the highest genetic distance (D_A) was observed between the *obtusa* and *globosa* varieties, while the minimum D_A was observed between the var. *acuta* and var. *globosa* (Chandra Shekar et al. 2016). Nevertheless, little information is available for the mitochondrial DNA of these commercially important morphological variants.

Microsatellites have become one of the most widely used genetic markers to infer population genetic structure (Bruford and Wayne 1993, Paetkau et al. 1995, Arularasan et al. 2014). But as gastropod females often mate with more than one male (Martel et al. 1986), there is likely an increase of nuclear genetic diversity within each area, hence mitochondrial DNA has proven to be powerful for genealogical and evolutionary studies in gastropods (Wethington et al. 2009). Among the mitochondrial markers, the 16S rRNA gene has become the most targeted for population dynamics studies and has proven successful for delineating divergent clades in many gastropod taxa (Parker et al. 1998, Holznagel and Lydeard 2000).

The present study was performed with the aim to examine the haplotype variation and phylogenetic patterns of the three commercially important morphological variants of *T. pyrum* from the southeast coast of India—var. *obtusa*, var. *acuta,* and var. *globosa*—using a molecular approach involving the mitochondrial 16S rRNA gene sequence data.

Materials and Methods

Sampling.—In total, 93 fresh specimens[1], identified based on their shell morphology (Hornell 1916), belonging to the three variants of genus *Turbinella,* were collected haphazardly from the southeast coast of India (Table 1). The samples were collected between December 2012 and July 2014 from the commercial fish landing centers nearby their respective natural habitats. A piece of muscular foot tissue was dissected aseptically from each specimen. The tissue samples were then preserved in TNES urea-buffer (Asahida et al. 1996) and stored at 4 °C until DNA extraction.

DNA Extraction, Amplification, and Sequencing.—Genomic DNA was extracted from a small piece of foot tissue using Qiagen's DNeasy tissue kit following the manufacturer's instructions. The quantity and quality of the isolated DNA were assessed using NanoDrop ND-1000 (Thermo Scientific) prior to storing the DNA at −80 °C. The concentrated DNA samples were then diluted (up to 40 ng μl^{-1}) to reach appropriate concentrations for PCR amplification.

[1] Chanks under *Turbinella* genus are not banned under the Wildlife (Protection) Act, 1972, Government of India (http://envfor.nic.in/legis/wildlife/so1197%28e%29.htm).

Table 1. Summary of sampling locations and number of chanks sampled for three commercially important morphological variants of genus *Turbinella* from the southeast coast of India.

Sampling sites	Latitude	Longitude	Number of samples
var. *obtusa*			
Mudasal Odai	11°29′07.74″N	79°46′28.10″E	11
Nagapattinam	10°45′37.94″N	79°50′57.82″E	16
var. *acuta*			
Rameshwaram	9°16′49.46″N	79°19′02.44″E	18
Punnaikayal	8°38′15.20″N	78°07′13.63″E	11
var. *globosa*			
Arogyapuram	8°07′10.76″N	77°33′32.25″E	14
Colachel	8°10′20.67″N	77°14′56.42″E	23

A segment of the mitochondrial 16S rRNA gene was amplified using universal primers (Palumbi 1996). PCR was conducted in 25 µl reaction volume containing 40 ng of each primer, 200 µM dNTP mix, 1.5 nM $MgCl_2$, 2.5 mM of 10× buffer, 100 ng DNA template, and 1U Taq DNA polymerase (Sigma). The PCR cycling parameters were optimized as follows: initial denaturation at 94°C for 5 min, followed by 94 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min for 30 cycles, and a final extension at 72 °C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis in 0.5× TBE buffer at 110 V and visualized using a gel documentation system (Gel Logic 100, Kodak).

After confirmation of proper amplification, the PCR amplicons of 16S rRNA were purified based on the silica-membrane method using QIAquick PCR Purification Kit, following the manufacturer's instructions. The purified PCR amplicons were quantified using NanoDrop ND-1000 (Thermo Scientific). PCR products were then cycle-sequenced using BigDye® Terminator v3.1 kit (Applied Biosystems) following the manufacturer's protocol with minor adjustments: all cycle sequencing reactions were carried out following the manufacturer's standard protocol (Applied Biosystem, USA) in a total volume of 20 µl, utilizing 1 µl (30–50 ng) PCR amplicon, 2 µl BigDye ready reaction mix, 1 µl of either forward or reverse primer (2.5 µM), 4 µl Sequencing Buffer (5×), and nuclease-free water. Further, following post-reaction clean-up, the amplicons of the 16S rRNA gene were sequenced using an automated DNA sequencer (ABI HITACHI 3500) following Sanger's dideoxy chain termination method (Sanger et al. 1977).

Data Analyses.—Individual 16S rRNA gene sequences were unambiguously assembled, edited, and aligned using SeqScape v2.5 (Applied Biosystem, USA) with default parameters. Sequences were submitted to GenBank under the accession numbers: var. *obtusa* (TPO) KJ409844–KJ409871, var. *acuta* (TPA) KJ409778–KJ409806, and var. *globosa* (TPG) KJ409807–KJ409843.

The population genetics program Arlequin v3.5 (Excoffier and Lischer 2010) was used to compute statistical parameters and tests. Effective numbers of haplotypes (*He*) were calculated using formula $He = 1/(1 - h)$, where h is the number of haplotypes (Jost 2007, Timmers et al. 2012).

The program MEGA v6 (Tamura et al. 2013) was used to study the evolutionary history and to generate a phylogenetic tree using the obtained sequences (i.e., prior to missing data curation). The evolutionary history was investigated using the

Table 2. Summary information and diversity statistics of studied chank varieties for three commercially important morphological variants of genus *Turbinella* from the southeast coast of India. *N*: sample sizes; N_l: number of positions; N_{ul}: number of usable positions with <5% missing data; N_p: number of polymorphic sites; N_h: number of haplotypes; *He*: number of effective haplotypes; *H*: haplotype diversity; π: nucleotide diversity; N_s: number of substitutions.

Parameters	Morphological variants			Mean (SD)
	var. *obtusa*	var. *acuta*	var. *globosa*	
N	28	29	37	
N_l	584	584	584	
N_{ul}	480	337	482	
N_p	54	56	28	
N_h	15	23	22	
He	4	25	10	
H	0.759	0.960	0.904	0.874 (0.104)
π	0.021	0.060	0.017	0.033 (0.024)
N_s	62	70	40	

neighbor-joining method (Saitou and Nei 1987), wherein distances were computed by Kimura 2-parameter (K2P) (Kimura 1980), and branch confidence was assessed via bootstrap analysis (1000 replicates).

Results

Sequence Diversity and Population Structure.—A 584-bp fragment was obtained for the 16S rRNA locus in all the investigated samples (93) including var. *obtusa* (27), var. *acuta* (29), and var. *globosa* (37). Among these sequences, a total of 60 haplotypes were observed. Distribution and relative frequencies of haplotypes among the three morphological variants of genus *Turbinella* are shown in Table S1. Among all the haplotypes under study, TPG26, TPG38, and TPG22 were the most common and were shared in both var. *acuta* and var. *globosa* populations. The common haplotype TPG26 was represented in 20.7% of sampled individuals in var. *acuta* and 21.6% of sampled individuals in var. *globosa*. TPG38 was represented in 3.5% of sampled individuals in var. *acuta* and 24.3% of sampled individuals in var. *globosa.* TPG22 was represented in 3.5% of sampled individuals in var. *acuta* and 2.7% of sampled individuals in var. *globosa*. In var. *obtusa* samples, the haplotype TPO30 was represented in 50% of sampled individuals and did not share any haplotype with the other two variants.

Standard and molecular diversity statistics were assessed (Table 2). Total amplicon size after excluding sequences missing from >5% of data was found to be 480 for var. *obtusa,* 337 for var. *acuta* and 482 for var. *globosa*. Overall, 138 polymorphic sites were observed, defining 60 haplotypes, with an overall haplotype diversity (*h*) of 0.874 (SE 0.104). Haplotype diversity varied between 0.759 (var. *obtusa*) and 0.960 (var. *acuta*). The effective number of haplotypes was found to be 4 for var. *obtusa*, 25 for var. *acuta,* and 10 for var. *globosa*. Nucleotide diversity (π) was observed to be the least for var. *globosa* (0.017) and highest for var. *acuta* (0.060), with an overall mean of 0.033 (SE 0.024). The var. *acuta* samples also showed a much higher number of substitutions (70) than that of var. *obtusa* (62) and var. *globosa* (40).

Slatkin's linearized F_{ST} and Reynolds' distance (R_{ST}) values between three studied morphological variants are presented in Table S2. The highest genetic distance

Table 3. Analysis of molecular variance results showing variance components for three commercially important morphological variants of genus *Turbinella* from the southeast coast of India. df = degrees of freedom, SS = sum of squares.

Source of variation	df	SS	Variance component	Percentage of variation
Among populations	2	455.094	7.080	48.50
Within populations	91	684.311	7.520	51.50
Total	93	1,139.404	14.601	100.00

(0.931) was found between var. *obtusa* and var. *globosa,* followed by 0.788 between var. *obtusa* and var. *acuta.* The least genetic distance (0.052) was found between var. *acuta* and var. *globosa*. Similarly, the highest (0.835) Reynolds' distance (R_{ST}) was observed between var. *obtusa* and var. *globosa,* followed by that between var. *obtusa* and var. *acuta* (0.687); the least (0.051) emerged between var. *acuta* and var. *globosa.*

The average number of pairwise differences (21.896) were highest between var. *obtusa* and var. *globosa,* and corrected average pairwise differences (43.427) were highest between var. *obtusa* and var. *acuta*. Corresponding lowest values of 0.851 and 21.544 were observed between var. *acuta* and var. *globosa* (Table S3). The average number of pairwise differences within var. *acuta* indicated lower (3.948) within-variant diversity vs var. *globosa* (4.438) and var. *obtusa* (6.455). Analysis of molecular variance (AMOVA) also revealed the highly significant percentage of variation (48.50%) among variants (Table 3).

Phylogenetic Analyses.—The relationships of three morphological variants under the genus *Turbinella*—var. *obtusa,* var. *acuta,* and var. *globosa*—based on 16S rRNA gene was determined using neighbor-joining (NJ analysis), and are shown in Figure 2 with respective bootstrap values indicated above each branch. The phylogenetic tree showed two main phylogroups, supported by the maximum (99%) bootstrap value: the first phylogroup that included only specimens of the var. *obtusa*. The phylogenetic tree supported by highest (99%) bootstrap value revealed that the specimens of var. *obtusa* formed a monophyletic clade. In contrast, the samples of var. *acuta* and var. *globosa* clustered into one separate monophyletic clade.

Discussion

Investigation of mitochondrial 16S rRNA gene sequences revealed considerable haplotype variation and phylogenetic patterning in the three studied morphological variants of the genus *Turbinella*.

Genetic Variation and Population Structure.—The unique haplotypes observed in the studied variants have value for species identification. Regarding population differentiation, the haplotype distribution (Table S1) revealed that the population var. *obtusa* was unique as it did not share any haplotype with the other two populations. However, few of the haplotypes were shared among the other two morphs that could imply their recent divergence, incomplete lineage sorting, or the prior taxonomic error (Chandra Shekar et al. 2016). Similar observations were also made in the case of molecular evolutionary studies in the endemic gastropods of the Lake Titicaca (Kroll et al. 2012).

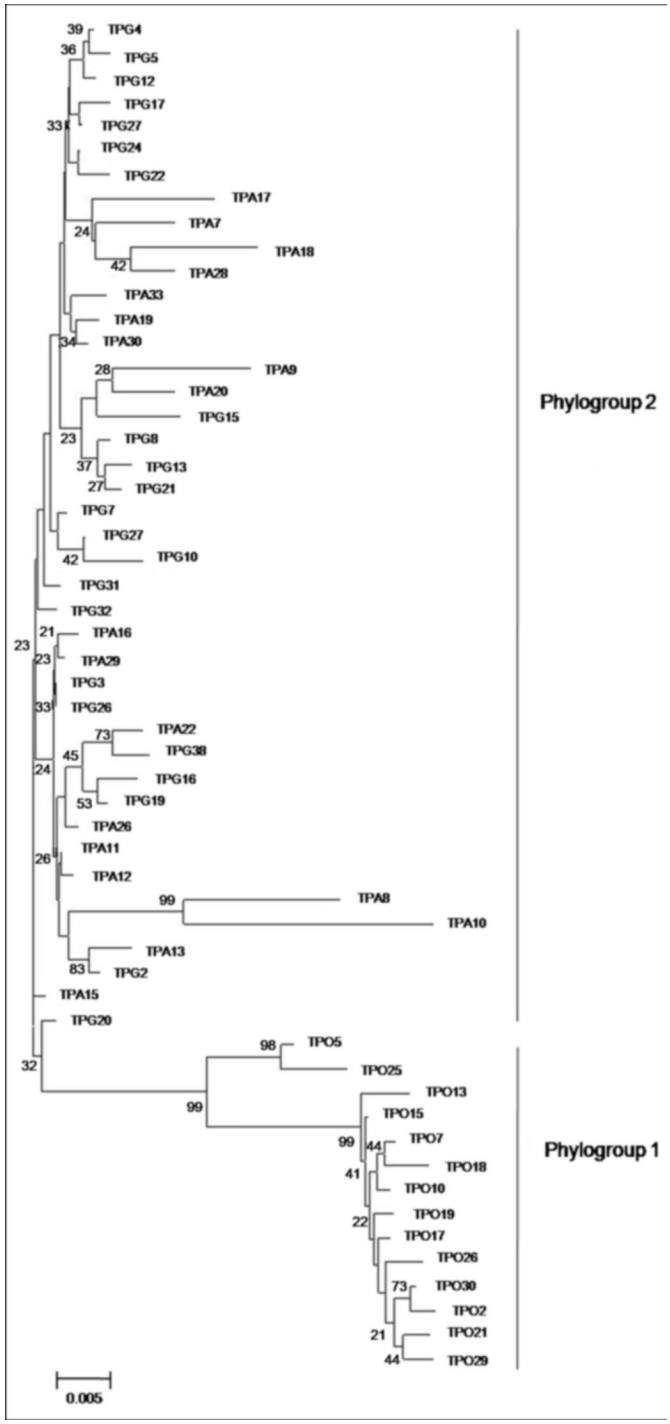

Figure 2. Evolutionary relationships of taxa using the Neighbor-Joining method. NJ tree for var. *obtusa* (TPO), var. *acuta* (TPA), and var. *globosa* (TPG); bootstrap values calculated from 1000 full heuristic replicates are shown next to the branches. The optimal tree with the sum of branch length = 0.53131312.

The present findings of standard diversity statistics showed that the genetic variation between studied chank varieties was highly significant. The amplicon fragment size found in the present investigation was slightly lower than the findings in *Lunella granulata* (Gmelin, 1791), a marine gastropod mollusk in the family Turbinidae (Chiu et al. 2013). Similar to our results, high numbers of haplotypes (30) were also observed in the geographically separated populations of *L. granulata* (Chiu et al. 2013, Yasuda et al. 2015). The total number of polymorphic sites found for the three varieties studied here are similar to the findings of *L. granulata* (48) (Chiu et al. 2013) and *Nacella magellanica* (Gmelin, 1791) of the family Nacellidae (58) (González-Wevar et al. 2012). In the present study, the haplotype diversity varied between 0.759 and 0.960, similar to the high haplotype diversity values reported in *L. granulata* (0.899) (Chiu et al. 2013). However, lower haplotype diversity in sampling localities for *Megastraea undosa* (W. Wood, 1828) (Turbinidae) has been reported (Haupt et al. 2013). Also, a low number of effective haplotypes have been reported in studied groups of *M. undosa* (Haupt et al. 2013). In contrast, the present study revealed high haplotype diversity. However, moderately low nucleotide diversity values were recorded for var. *obtusa* and var. *acuta,* while it was much lower (0.017) for var. *globosa*. The lower nucleotide diversity in the studied variants reflected that the differences between haplotypes are mostly of a single nucleotide. Likewise, similar nucleotide diversity value of 0.75 was reported for *Nassarius nitidus* (Jeffreys, 1867) (Gastropoda, Nassariidae) (Albaina et al. 2012) and much lower nucleotide diversity (0.00288) in *N. magellanica* (González-Wevar et al. 2012).

The significant F_{ST} and R_{ST} values recorded in present study correspond to the particular geographical distribution of studied variants. All F_{ST} values for pairs of variants obtained in the present study indicate absolute differentiation within their genetic structure. Moreover, comparatively higher values of an average number of pairwise differences and corrected average pairwise differences in var. *obtusa* indicated greater evolutionary divergence from the other two studied variants. The average genetic differences obtained in the present study were also correlating with the geographic distribution (Fig. 1) for the whole set of samples of the three studied variants of *Turbinella*. Due to lack of reported data from previous studies on pairwise differences of other species of the genus, the direct comparison with nearest genetic groups cannot be carried out. AMOVA analyses revealed that the variance components among variants were highly significant for the studied 16S rRNA locus (Table 3), demonstrating distinct genetic composition in the chank varieties examined. Many factors, such as species mobility, ocean currents, historical variance, and geographic distance, influence the development of population structure in a marine environment (Saarman et al. 2010). The possible explanation for the heterogeneity in the three studied variants could be the low level of gene flow. The slow migration rate and small breeding territories for marine gastropods might also be one of the reasons for distinct population structure in the three variants of *Turbinella*. Similar to the present findings, Chiu et al. (2013) examined genetic variance in *L. granulata* and found that nearly 71.31% of genetic variation prevailed among geographic zones and 3.49% among samples within the geographic area, while within populations it was 25.2%.

Phylogeny.—Our neighbor-joining analyses results indicated a major phylogenetic split (Fig. 2), and the overall geographical concordance of the phylogroups supports the earlier classification of the studied morphological variants of *Turbinella* (Winckworth 1939). As observed, the var. *obtusa* samples showed distinct genetic composition, which might be due to its geographical isolation from the other two variants under study. Based on our findings and from the genetic perspective of the 16S rRNA gene, it is possible that var. *obtusa* was an independent and highly differentiated population among the three studied variants. Despite the fact that the samples of var. *acuta* and var. *globosa* clustered into a monophyletic clade, they were not reciprocally monophyletic based on the substantial intrapopulation variation as revealed by our diversity metrics. However, owing to their geographic proximity, there was a chance of admixture due to gene flow between these two populations (Chandra Shekar et al. 2016).

In summary, our results based on the 16S rRNA gene sequence analyses support the earlier taxonomic classification of *T. pyrum* var. *acuta* as a separate species, i.e., *T. rapa* described by Winckworth (1939), further providing a preliminary genetic basis for more in-depth studies using other hypervariable and highly conserved mitochondrial regions. Also, the present findings strongly suggest consideration of genetic variation among the studied morphological variants of the genus *Turbinella* when contemplating management and/or conservation strategies.

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