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Application of Mitochondrial DNA Polymorphism to *Meloidogyne* Molecular Population Biology[1]

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Abstract: Recent advances in molecular biology have enabled the genotyping of individual nematodes, facilitating the analysis of genetic variability within and among plant-pathogenic nematode isolates. This review first describes representative examples of how RFLP, RAPD, AFLP, and DNA sequence analysis have been employed to describe populations of several phytonematodes, including the pinewood, burrowing, root-knot, and cyst nematodes. The second portion of this paper evaluates the utility of a size-variable mitochondrial DNA locus to examine the genetic structure of *Meloidogyne* isolates using two alternate methodologies, variable number tandem repeat (VNTR) and repeat associated polymorphism (RAP) analysis. VNTR analysis has revealed genetic variation among individual nematodes, whereas RAP may provide useful markers for species and population differentiation.

Key words: AFLP, genetics, mitochondrial DNA, molecular biology, molecular markers, nuclear DNA, PCR, RAP, RFLP, root-knot nematode, VNTR.

Why study the genetic structure of nematode populations? Although this area remains the least understood aspect of nematode population biology (8), quantitative description of genetic variability among nematode populations must now assume a pivotal role in contemporary phytonematology. The escalating reliance on environmentally friendly, non-nematicidal management strategies including plant-host resistance, crop rotation, and biological control are measures that likely involve genotypic interactions between the plant nematode and its host. As a consequence, the selection and efficacy of appropriate control strategies will be impacted by the genetic variation that defines targeted nematode populations. We must learn of the genetic differentiation that underlies populations and address whether standard management regimes are controlling only a portion of the component subpopulations.

Within traditionally defined taxonomic groups, the variable host ranges of closely related nematodes have generated descriptions of sibling species and subspecific races or pathotypes (13,26). These subtle, operational phenotypes may be a direct consequence of the selection and fitness of various genotypes that collectively comprise nematode field populations. Characterization of the genetic differences propelling differentiation at the subspecific level represents a major challenge in nematode population and evolutionary biology, ecology, and management.

How is genetic variability currently assessed within phytonematode populations? In the past, protein profiles and serological techniques have provided insight into taxonomic relationships and species boundaries (22). Because phytonematodes are small, these methodologies lacked the sensitivity to genotype individuals, severely limiting the genetic characterization of populations.

Contemporary techniques of molecular biology, including molecular cloning, polymerase chain reaction (PCR) amplification, and nucleic acid sequencing, have alleviated earlier methodological difficulties that impeded measurement of genetic variability among nematode populations (9,23,25). Moreover, DNA analysis provides a direct and more sensitive measure of genetic variability because protein phenotypes result from a small fraction of the genome and genotypic changes that do not produce electrophoretic variants remain undetected.

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Nucleic acid sequencing provides the most sensitive measure of genetic variation because single base substitutions are revealed. In conjunction with molecular cloning or PCR amplification, homologous portions of the genome from multiple individuals can be isolated, and sequences aligned and compared. This information can be used to measure heterozygosity within populations and deduce phylogenetic affinities. Recent advances in direct sequencing of PCR-generated DNA fragments have obviated the absolute requirement for isolation of target genes by molecular cloning, thereby permitting a larger number of samples and, hence, loci to be assessed.

Bursaphelenchus xylophilus and *B. mucronatus* (2) were studied using nucleotide sequence analysis. By scoring the occurrence of base substitutions within the PCR-amplified heat shock 70A gene, five different *B. xylophilus* sequences were obtained from 11 populations. Within *B. mucronatus* isolates, four sequence variants were detected among eight isolates.

Despite advances, sequencing can be a labor-intensive effort. To expose genetic variability within and among closely related populations, it will be necessary to survey larger numbers of individual nematodes and a more expansive fraction of the genome, a requirement rendered impractical by DNA sequencing alone. To circumvent this problem, alternate DNA methodologies (9) have been adapted to study nematode population differentiation; these include restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis.

RFLP identifies limited nucleotide substitutions and DNA rearrangement via isolate-specific changes of DNA fragment size generated by restriction enzyme digestion. In phytonematodes, one strategy has been to target highly repeated nuclear DNA elements within the nematode genome and score the changes among the repeats (6,30). Highly reiterated DNA sequences, or "satellite" DNAs, often contain no coding information. These DNA segments are free to change faster than coding regions because they are not subject to selection pressure that limits nucleotide variation and, hence, restriction site polymorphism in functional loci.

Repetitive DNA sequences cloned from the *Meloidogyne incognita* nuclear genome (6) were used to estimate RFLP genetic distances among root-knot nematode species and populations (7); phylogenetic affinities deduced in this study were consistent with clustering based on modes of reproduction, and suggested amphimixis as an ancestral reproductive strategy. Similar approaches using repetitive DNA (5) and the intergenic rDNA spacer regions (20) revealed variation within and among *M. arenaria* race 2 isolates. Surprisingly, cDNA clones demonstrated an elevated level of RFLP not anticipated for functional genes among these same race 2 isolates (20).

RFLP typing of *Meloidogyne* mitochondrial DNA (mtDNA) was employed to describe Australian root-knot nematode populations (21). This study examined 64 samples representing four major root-knot nematode species. Six abundant mitochondrial DNA (mtDNA) classes (termed haplotypes) were carried among the *M. arenaria, M. javanica,* and *M. incognita* isolates examined. Despite the relatively low genetic variation among the mitochondrial genomes of the three species (less than 0.6% sequence divergence as estimated by RFLP occurrence), there was a correlation between mtDNA haplotype and species designation, and clustering of mtDNA haplotypes derived from reproductively isolated but geographically proximate populations. *Meloidogyne hapla* appears to contain multiple mtDNA haplotypes (29), each differing substantially from the three other root-knot nematode species analyzed (21).

RFLP and DNA sequence analysis also has been used to examine relationships among cyst nematode isolates. MtDNA RFLP revealed a separation of the sibling species *Heterodera glycines* and *H. schachtii* 7.3 to 14.8 million years ago (33). Nucle-

otide sequence analysis of the rapidly evolving internal transcribed rDNA spacers (ITS) has revealed strong affinities among representative species of the *schachtii* group (*H. glycines, H. schachtii,* and *H. trifolii*), though some differentiation among soybean cyst nematode isolates could be scored (14). Substantial sequence divergence has also been reported between *H. carotae* and *H. avenae* as well as the *schachtii* group nematodes (15). As with *H. glycines,* there is considerable conservation in ITS among geographic isolates of *H. avenae,* with the exception of the Gotland strain pathotype.

Species and sub-specific genetic variation can be exposed by RFLP and nucleotide sequence analysis, even though a relatively small portion of the genome is targeted by these strategies. To increase the probability of revealing divergence within and among closely related phytonematode populations, RAPD technology has become increasingly popular (19). This technique employs PCR to amplify randomly targeted portions of the genome. PCR-generated fragments, visualized as distinctive banding patterns after electrophoretic fractionation, are used as genetic markers.

RAPD analysis has revealed a highly conserved genome organization between two sibling species of the burrowing nematodes, *Radopholus citrophilus* and *R. similis* (24). This result stands in contrast to the substantial genetic variation observed when RAPD approaches have been applied to cyst and root-knot nematode isolates. Patterned arrays of PCR-amplified DNA fragments were sufficiently different to allow discrimination between California populations of *H. cruciferiae* and *H. glycines* (10). Importantly, variability among electrophoretic banding patterns representing six *H. schachtii* isolates also was scored. Lineages that contributed to variability within populations could be identified by amplification using template DNA derived from single cysts. A similar approach has been used to estimate genetic differences among root-knot nematode species (18) and also has found utility in differentiating *M. hapla* races A and B and two separate *M. arenaria* race 1 root-knot nematode isolates (11).

RAPD analysis has revealed genetic differentiation between the potato cyst nematode (PCN) sibling species *G. rostochiensis* and *Globodera pallida* (34) and between pathotypes of *G. rostochiensis* (12). Amplified fragment length polymorphism (AFLP), a refined amplification technology capable of fingerprinting a larger portion of the genome, has revealed considerable genetic variation among PCN isolates (17). Cluster analysis of *G. rostochiensis* RAPD patterns (16) and AFLP types (17) has revealed coincidence with pathotypes. In contrast, affinities deduced from RAPD and AFLP variation among *G. pallida* isolates did not conform to host range, suggesting the pathotype classification scheme may require redefinition.

Both RFLP and RAPD analyses have confirmed the anticipated genetic diversity of phytonematodes at the sub-specific level. We now evaluate two relatively new approaches that may be useful in studying the genetic structure of nematode populations, resolving genetic affinities among nematode isolates, and elucidating patterns of gene flow.

Size-variable mtDNA analysis of Meloidogyne *populations:* In the following sections, two types of mtDNA analysis are described. Genetic differentiation among root-knot nematode isolates may be responsible for the variable efficacies of different nematode control tactics (27), and the *Meloidogyne* mitochondrial genome appears particularly amenable to population genetic analysis using the approaches detailed below.

MtDNA heteroplasmy is defined as the presence of two or more distinguishable mitochondrial genome types within an individual. One form of heteroplasmy involves mtDNA molecules of different sizes resulting from copy number differences among tandemly repeated sequences (termed variable number tandem repeats, or VNTRs). *Meloidogyne* spp. mtDNA contains three separate sets of short VNTRs

composed of 8, 63, and 102 base pair repeating units (28). These loci have been observed in *M. javanica, M. hapla* race A, *M. incognita* races 1 and 4, and *M. arenaria* race 2 (28).

We have developed a PCR-based assay to identify and catalog variation in copy number of the *Meloidogyne* 63 bp VNTR. Based on available nucleotide sequence information for unique mtDNA sequences that flank the *M. javanica* 63-bp VNTR (28), synthetic oligonucleotide primers were designed to amplify this mtDNA region from crude nematode lysates. DNA preparations prepared from approximately 10^4 nematodes representing three *M. javanica* isolates (from Riverside, CA and Lincoln, NE) and an *M. incognita* race 1 were used as PCR templates. When the resultant PCR products were resolved (Fig. 1), the DNA fragments appeared as ladders with each "rung" 63 bp larger then the adjacent, lower-molecular-weight neighbor. This result indicated that the PCR products were generated from a mixture of mtDNA template molecules that differed from each other by the precise copy number of 63 bp repeating units, and that a number of size-variant mitochondrial genome molecules were maintained in these *Meloidogyne* populations. However, because DNA was pooled from numerous J2 and adults, how mtDNA forms were distributed among individuals could not be determined.

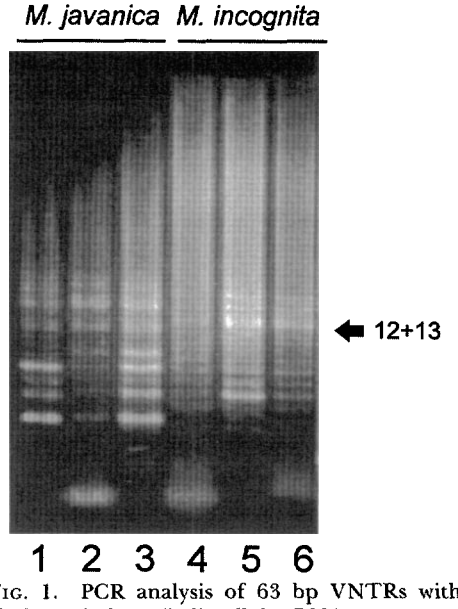

FIG. 1. PCR analysis of 63 bp VNTRs within *Meloidogyne* isolates. Bulk cellular DNA was prepared from approximately 10^4 mixed second-stage juvenile and adult-stage nematodes and used as template for PCR amplification with primers that bind external to the tandem array. PCR products were fractionated on a 1.2% agarose gel and visualized by ethidium bromide. (→) slightly resolved doublet representing alleles containing 12 and 13 repeat copies. Lanes 1 to 3, *M. javanica* isolates PRJC2, PRJC17, and E781 (500-ng template concentrations); lanes 4 to 6, *M. incognita* race 1 at DNA template concentrations of 5, 50, and 500 ng. Amplification conditions: 35 cycles at 94 °C, 40 seconds; 45 °C, 80 seconds; 72 °C, 90 seconds.

Fluorescent intensity of the amplification products provides an indicator of the relative frequency for each size variant in these bulk preparations. Qualitative differences in the abundance of mitochondrial genomes housing different 63 bp repeat copy numbers are apparent when the *M. javanica* and *M. incognita* PCR products are compared (Fig. 1).

When DNA from individual nematodes is used as template for PCR, a different electrophoretic profile of 63 bp VNTR alleles is obtained. Each individual contains only a subset of the alleles found in the population. For example, the five *M. incognita* individuals genotyped in Fig. 2A maintain only mtDNAs with VNTRs containing 12, 13, and 15 copies of the 63 bp repeat. Importantly, among the 30 individuals analyzed from this population, including the five depicted in Fig. 2, the VNTR containing 12 repeats was most common, consistent with the most frequent size VNTR amplified from the bulk *M. incognita* preparation (Fig. 1, lanes 4–6). That PCR amplification may be used to depict relative allele frequencies is supported by this observation. Diversity among individuals within this population is demonstrated by the variation in fluorescent intensities of the 12, 13, and 15 copy repeats when banding profiles derived from individual nematodes are compared (Figs. 2A,B).

Genetic diversity among root-knot

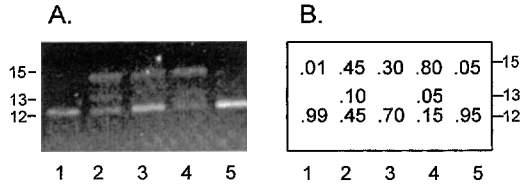

FIG. 2. 63 bp VNTRs within sibling *M. incognita* race 1 individuals. A) Total cellular DNA was isolated from five individual eggs housed in the same egg mass. PCR-amplified alleles generated as described in Fig. 1 were fractionated on a 1.5% agarose gel and visualized by ethidium bromide. Numbered gel lanes (1 to 5) designate individual nematodes. VNTRs containing 12, 13, and 15 copies are labeled. B) Densitometric analysis of allele frequency. Fluorescent intensities depicted in (A) were measured using ImageQuant (Molecular Dynamics). Values, substituted for DNA bands in (A) indicate the relative frequency of each allele within an individual nematode and are used for calculation of K indices (Table 1).

nematode populations can be quantitatively addressed by measuring the relative frequencies of the 63 bp VNTR as distributed among individuals. Average size class frequencies within isolates can be calculated by summing the frequencies for a size class among individuals and dividing by the total number of nematodes sampled.

Frequencies of size-variant mtDNA molecules can be statistically apportioned into variation within individuals, among individuals within a local population, and among isolates (3). This treatment predicts the probability with which two identically sized molecules (containing precisely the same copy number of a repeated sequence) can be sampled from the hierarchical levels defined above; the greater the size diversity, the less likely two molecules containing the same VNTR copy number will be chosen from a mixture of mtDNAs. These probabilities are termed diversity (or K) indices. K indices are calculated from:

$$K = 1 - \sum_{n=0}^{n} x_i^2$$

where x_i represents the frequency for the "ith" mtDNA size class in the sample. For example, if the collection of mtDNAs all carry the same-sized 63 bp VNTR, $x_i = 1$ and $K = 0$, meaning that there is no genetic variation at this locus.

The K-indices for different types of samples are indicated by a letter subscript:

K_b = mtDNA diversity within an individual

K_c = mtDNA diversity within a group (deme, population)

K_d = mtDNA diversity among groups

K_b is calculated directly from size class frequencies within each individual; K_c and K_d can be obtained directly from the mean size class frequencies computed from population summations (Table 1).

We are interested in determining if the collection of mtDNA VNTRs is more diverse within individuals, between individuals within populations, or among populations. To elucidate the distribution of mtDNA VNTRs among levels of resolution, genetic diversity (as defined by K in-

TABLE 1. Hierarchical analysis of 63 bp bp VNTR diversity in a single *M. incognita* egg mass.

	Repeat number[a]						
Individual	12	13	15	K_b	K_c	C_i[b]	C_{ip}[b]
1	0.99		0.01	0.02			
2	0.45	0.10	0.45	0.59			
3	0.70		0.30	0.42			
4	0.15	0.05	0.80	0.34			
5	0.95		0.05	0.10			
Pool	0.65	0.03	0.32	x = 0.29	0.47		
						0.62	0.38

[a] Values represent densitometrically determined VNTR frequencies as in Fig. 2.
[b] C_i = average K_b/K_c; C_{ip} = 1 − average K_b/K_c.

dices) can be apportioned into components, or *C* values (3) (Table 1), where:

C_i = % total genetic diversity within individuals

C_{ip} = % total genetic diversity between individuals within a population

C_{pt} = % total genetic diversity between populations

and, by definition, $C_i + C_{ip} + C_{pt} = 1.0$.

An example is illustrated in Fig. 2A,B and Table 1. This "model" isolate represents the size-variable 63 bp locus amplified from five sibling J2-stage *M. incognita* individuals isolated from the same egg mass (Fig. 2A). Each lane represents alleles present in a single nematode. Based on densitometric analysis of the bands (Fig. 2B) and computation of *K* indices (Table 1), we find that when genetic variation of the 63 bp VNTR is apportioned into hierarchical levels (*C*-values), 62% of the diversity is found within individuals whereas 38% is found among nematodes that comprise this egg mass. Simply put, a more diverse spectrum of mitochondrial genomes occurs within a single J2 than among the individuals in the egg mass.

We also are evaluating the use of repeat-associated polymorphisms (RAPs) as another tool to examine relationships among root-knot nematode populations (31). The RAPs are point mutations (nucleotide substitutions) within or near tandemly repeated sequences, and they may occur at an elevated frequency in these portions of the mitochondrial genome. As such, RAPs may provide resolution that reveals affinities among species and populations.

Our search for RAP substitutions initially focused on mtDNA sequences within 63 bp VNTRs and flanking single copy regions. PCR-amplified size-variable VNTR alleles generated from bulked template DNA, as in Fig. 1, were isolated by molecular cloning into suitable bacterial vectors (pCRII, InVitrogen) and nucleotide sequences of at least two independent clones representing different VNTR sizes determined by "di-deoxy" chain termination (35). Sequence information that combines our studies on this mtDNA locus from *M. incognita*, *M. javanica*, and *M. arenaria* pop-

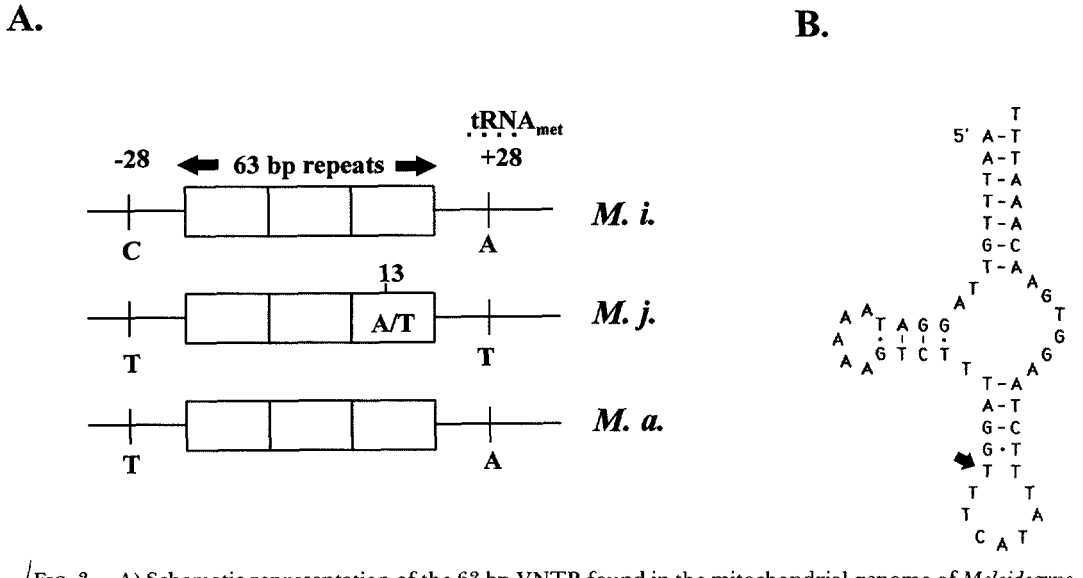

FIG. 3. A) Schematic representation of the 63 bp VNTR found in the mitochondrial genome of *Meloidogyne* isolates. Boxes represent individual 63 bp tandem repeating units. Numbers refer to specific positions occupied by variable nucleotides upstream (−), downstream (+), or within a tandem array. (. . . .), location of the tRNA$_{met}$ gene. *Mi* = *M. incognita*, *Mj* = *M. javanica*, *Ma* = *M. arenaria*. DNA sequence variation presented here was confirmed in at least two independent recombinant clones from each species isolate. B) Structure of tRNA$_{met}$ (adapted from 28); (→) +28 position of the RAP mutation in the anticodon stem region of the *M. javanica* mitochondrial tRNA$_{met}$ gene.

ulations are shown in Fig. 3A. Compilation of our data in this fashion revealed several observations that support the potential use of RAPs as a measure of species and population differentiation.

Species differentiation: Two nucleotide positions within the flanking regions of the 63 bp array revealed species-specific substitution (Fig. 3A). A T→C transition within a single *M. incognita* isolate was observed in the leftward flank, 28 bases upstream of the beginning of the first 63 bp repeating unit (−28). Another substitution, an A→T transversion, was observed at position +28 within 3′ flanking region. This location is of interest because it resides within the coding region for a mitochondrial-specific methionine tRNA ($tRNA_{met}$) that defines the 5′ end of the anticodon loop (Fig. 3B). In *M. javanica,* the T at this position is unpaired and creates a loop of nine nucleotides (including the anticodon triplet CAT), whereas the A at +28 characteristic of the *M. incognita* and *M. arenaria* $tRNA_{met}$ genes would hydrogen bond with T at position 36, creating a smaller seven-base anticodon loop. Mitochondrial gene sequences are typically conserved among closely related species, but the substitution described does not appear to alter $tRNA_{met}$ structure or function.

Population differentiation: Recombinant clones containing VNTRs derived from *Meloidogyne javanica* strain PRJC2, a virulent isolate from cowpea, contain an A at position 13 of the terminal 63 bp repeating unit that defines the rightward end of the VNTR (Fig. 3A). The published sequence by Okimoto et al. (28) reveals a T in the identical position within mtDNA molecules from *M. javanica* isolate NCSU no. 7-2. This substitution may establish a fixed nucleotide substitution between these two isolates, suggesting that mtDNA RAP analysis might also provide useful markers for population variability.

Summary and Conclusions

Although the differential host status of certain plants has revealed genetic diversity within and among root-knot nematode populations, only limited research has quantitatively addressed genetic variability among *Meloidogyne* isolates at the molecular level. Yet, with increasing reliance on non-chemical management practices, the ability to recognize and measure genetic diversity becomes essential.

The plasticity of tandem repeat arrays found within the root-knot nematode mitochondrial genome may provide a useful collection of markers to assess genetic variation within and among *Meloidogyne* isolates. We have begun to evaluate VNTRs and RAPs as possible approaches for detecting and quantifying genetic differentiation among root-knot nematode isolates. We are encouraged that the initial data described here have identified variability in VNTR copy number and in repeat-associated nucleotide polymorphism. However, these results have been obtained from a small number of mtDNA PCR products using a "model" isolate of small sample size (N = 5).

Several recurrent themes describing phytonematode population variability may be extracted from the recent literature and from the mtDNA results presented here:

(i) Population differentiation can be identified by using a variety of approaches including nuclear and mitochondrial RFLP, RAPD, and VNTR/RAP analysis.

(ii) When quantitative treatment of size-variable mtDNA alleles has been applied to populations of widely separated taxa, including insect (32), fish (1), and phytonematodes, there is striking within-individual and within-population variation. In particular, our early results with *Meloidogyne* VNTR analysis are consistent with the population structure found in the animal parasitic nematode *Ostertagia ostertagi,* where an mtDNA RFLP approach was employed (4). Excessive levels of within-individual variability will likely to be useful for studies addressing the sub-structure of com-

plex populations comprised of multiple-genetic lineages.

(iii) Where attempts have been made to deduce phylogenetic relationships among conspecific isolates (10,21), geographic proximity does not always agree with genetic affinity.

We anticipate that basic information addressing phytonematode population structure at the molecular level will ultimately have value to growers and plant nematologists who are eager to implement reliable and environmentally sound management practices.

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