

Evaluation of *cox1* and ribosomal RNA gene primers for DNA barcoding the phylum, **Platyhelminthes**

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Platyhelminthes (flatworms)

Classes:

Trematoda

(**flukes**)



Cestoda

(**tapeworms**)



Monogenea



Turbellaria

(**free-living**)



100,000
species?

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Sequences from diverse flatworms were obtained from GenBank and aligned.

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T. asiaticaA    C  ATGAGTGTAAATTTTGTAAAGTTGAATATTTACTTTAGATCATAAGCGGGTTGGTGTG
T. saginata    C  .....A..
T. solium      C  .....C....A.....G.....A.....A.A
T. crassiceps  C  ....CGT...GTGCA..TA.G....GT.G.....T..A..A..
E. granulosus C  ....A..GGTG.GA..AGGT..A..GG.G.....CA.A.....
H. diminuta    C  -----G.....TA...C...
S. mansoni     T  -----GGA.AT..TCA...GT.TG..T.....TA.A.....A
P. westermani T  -----A...T.C...GT.G..C.....G...C...TA.C..GA..
F. hepatica    T  -----A.G....GT.G....G..G.....T.....T..
  
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Degenerate cox1 Forward Primer

TTTWCITTRGATCATAAG

Degenerate cox1 Reverse Primer

TGAAAYAAAYAIIGGATCICCACC

Degenerate F and R cox1 primers with M13 “tails” were designed to amplify a broad array of flatworm groups.

DNA was extracted from samples of
Cestodes and Trematodes
at the CCDB using the glass fiber
extraction technique described in
Ivanova et al. Mol.Ecol.Notes 6:998-1002.

2 μ l of the resulting DNA sample was
amplified in a 25 μ l PCR reaction.

To determine whether DNA extractions were successful, samples were first amplified with 18S rRNA gene primers that produce a ~400 nt fragment from the 5' end of the gene.

To determine the utility of the cox1 primers for high-throughput barcoding, the PCR products were not cleaned prior to sequencing with M13F and M13R primers.

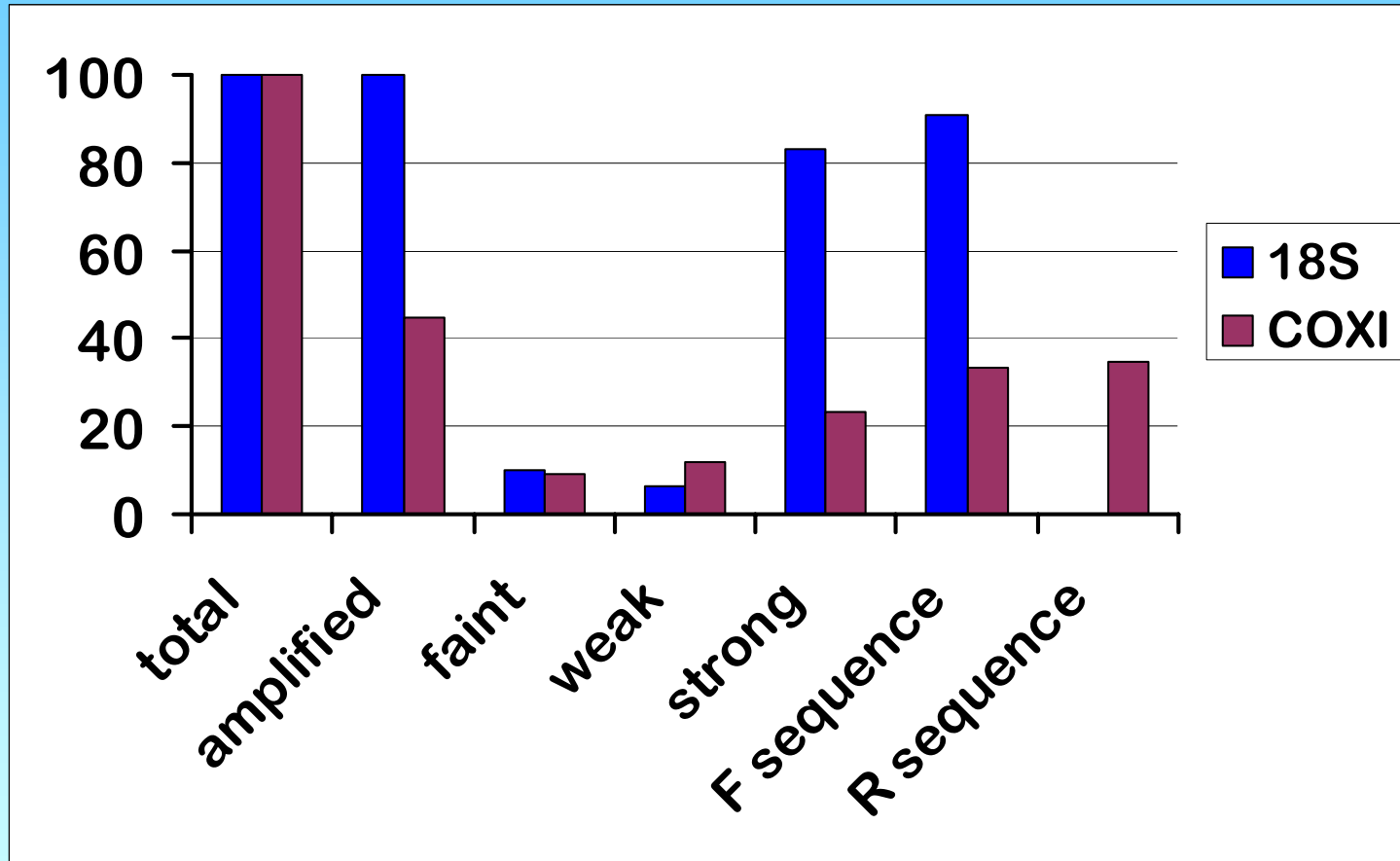
Both cestodes and trematodes amplified with the cox1 primers. Amplifications were not successful in all species or all specimens within species.

**At least one specimen amplified
and was sequenced in:**

2 species in 1 family of Cestodes

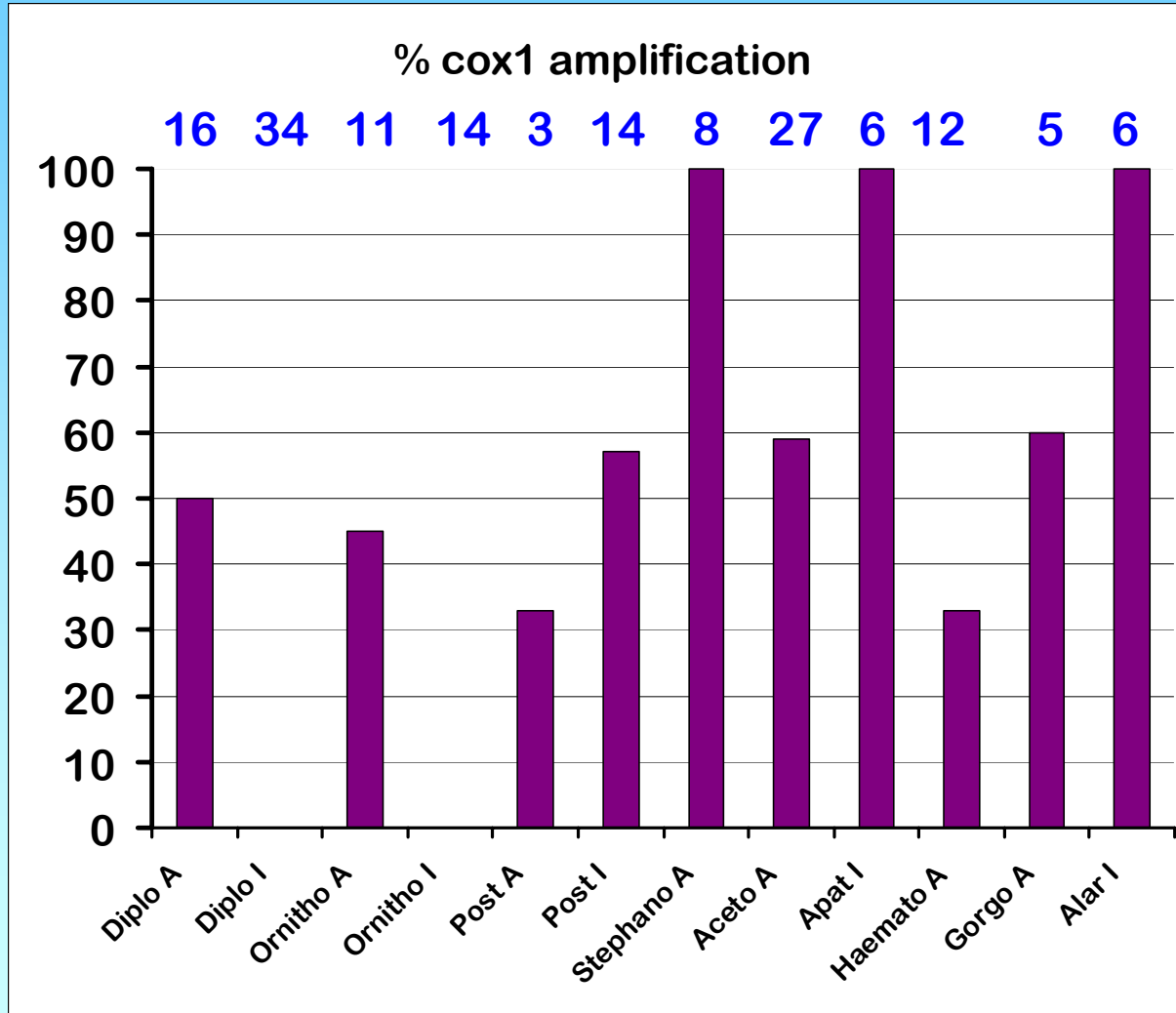
9 species in 6 families of Trematodes

Amplification success was higher using 18S rRNA than cox1 primers.

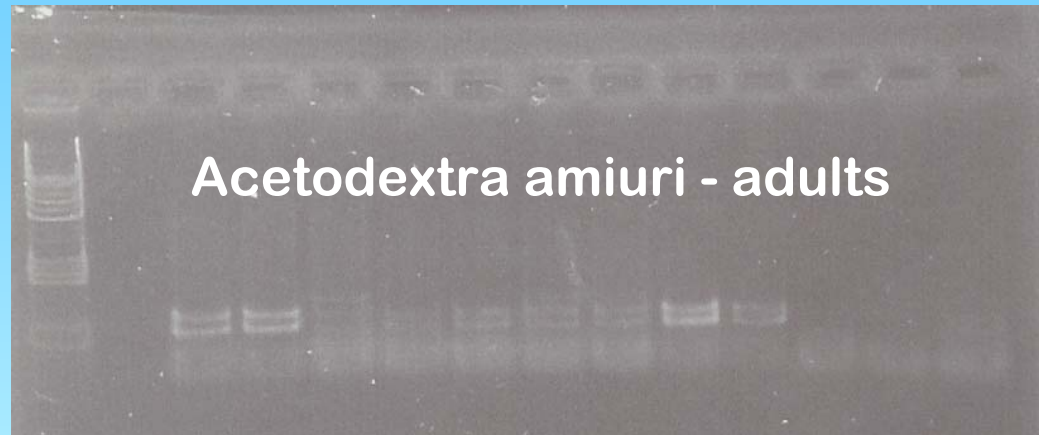


141 trematode samples

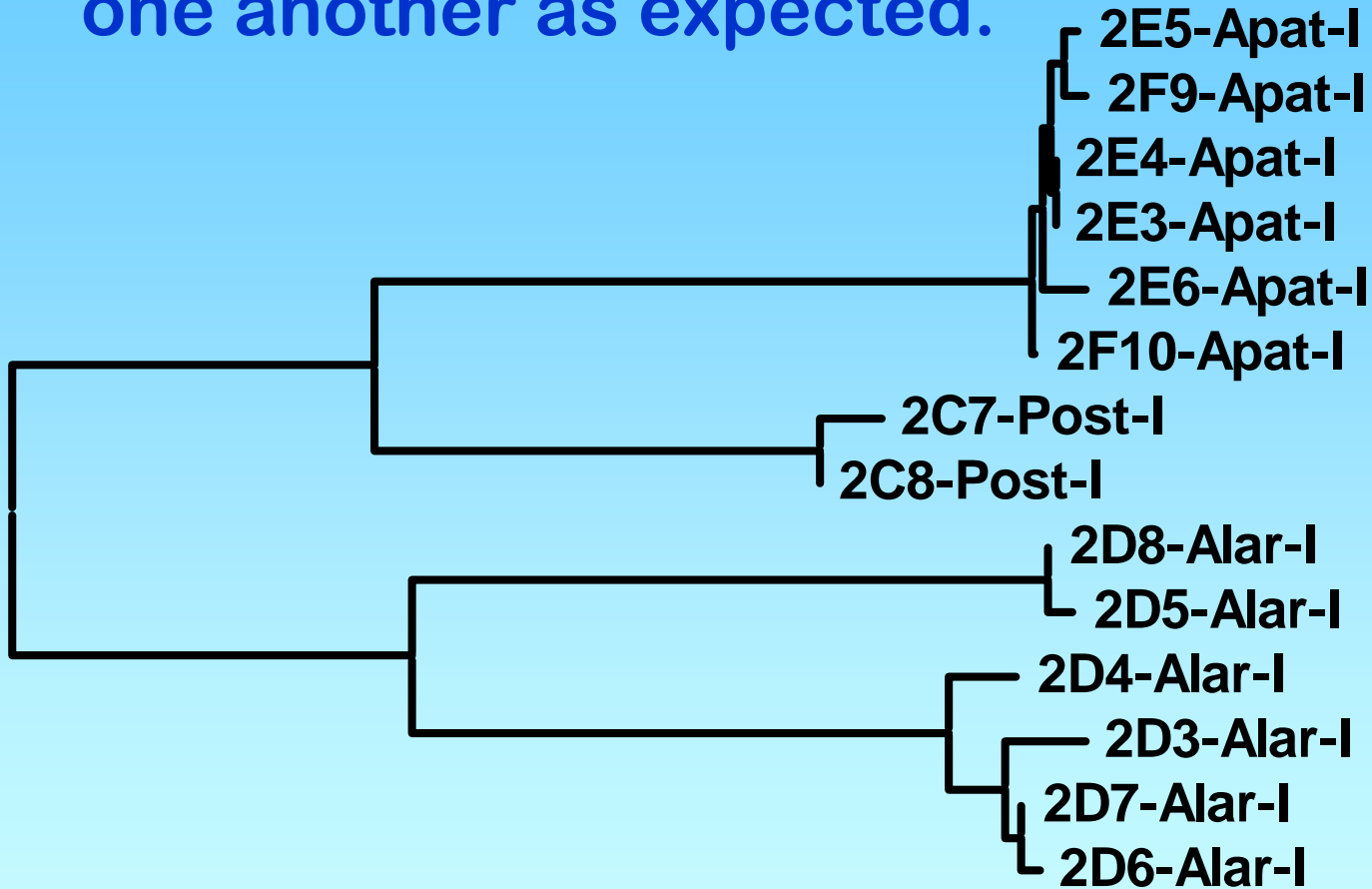
Amplification success varied among genera and life stage of trematodes



Amplification of cox1 from trematode genomic DNA



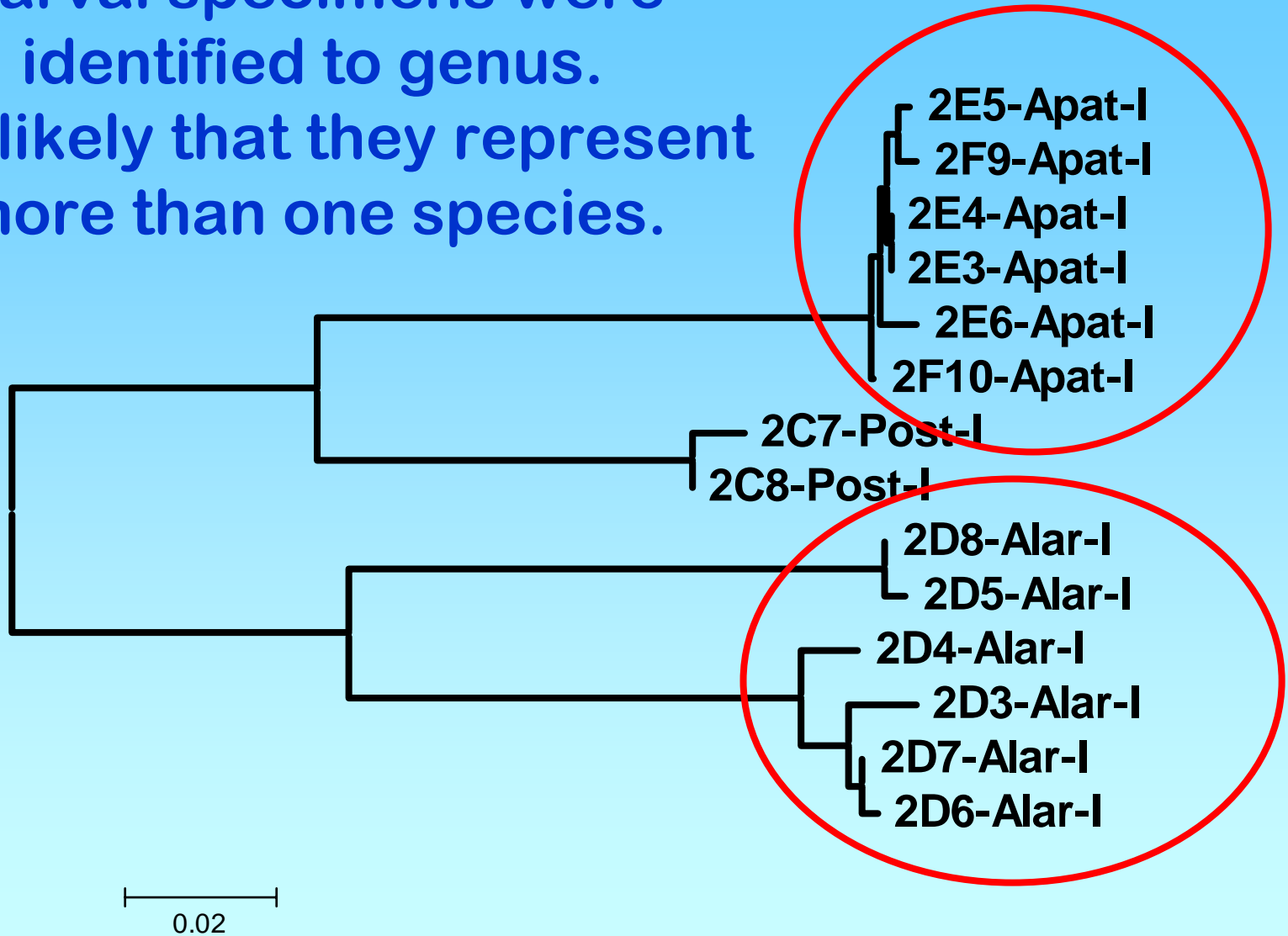
Genera vary substantially from one another as expected.



0.02

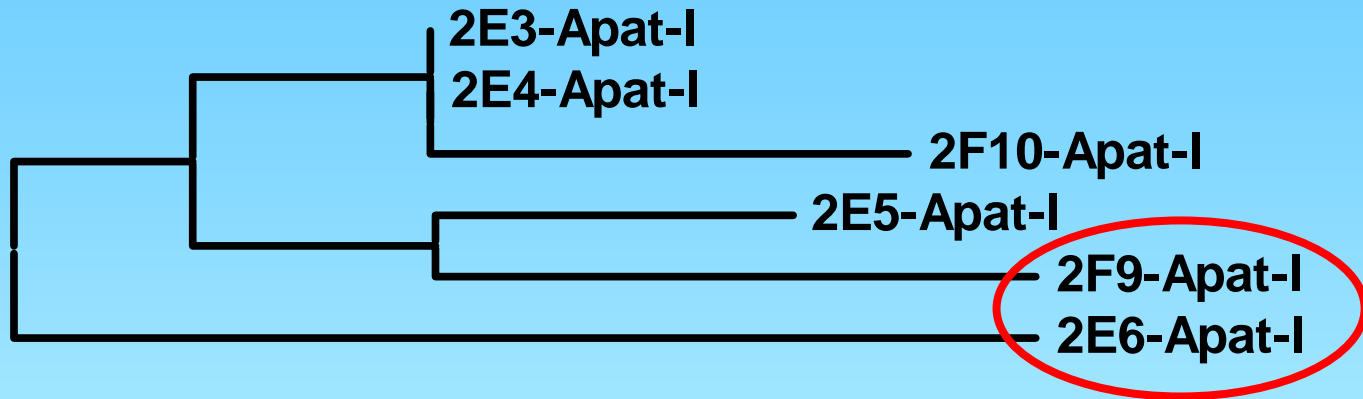
Box 1472 cox1F

Larval specimens were identified to genus.
It is likely that they represent more than one species.



Box 1472 cox1F

Cox1 identifies several distinct groups in the *Apatemon* larval specimens, but sequence divergence is <1%



	1	2	3	4	5
[1]2E3 -Apat-I	1				
[2]2E4 -Apat-I	0.000				
[3]2F10-Apat-I	0.002	0.002			
[4]2E5 -Apat-I	0.004	0.004	0.006		
[5]2F9 -Apat-I	0.004	0.004	0.006	0.004	
[6]2E6 -Apat-I	0.006	0.006	0.009	0.006	0.011

Cox1 sequences suggest that there are at least 3 species in the Alariinae larval samples.



0.01

	1	2	3	4	5
[1]2D6-Alar-I	1				
[2]2D7-Alar-I	0.000				
[3]2D3-Alar-I	0.011	0.011			
[4]2D4-Alar-I	0.020	0.018	0.020		
[5]2D5-Alar-I	0.145	0.142	0.148	0.142	
[6]2D8-Alar-I	0.141	0.137	0.143	0.137	0.002

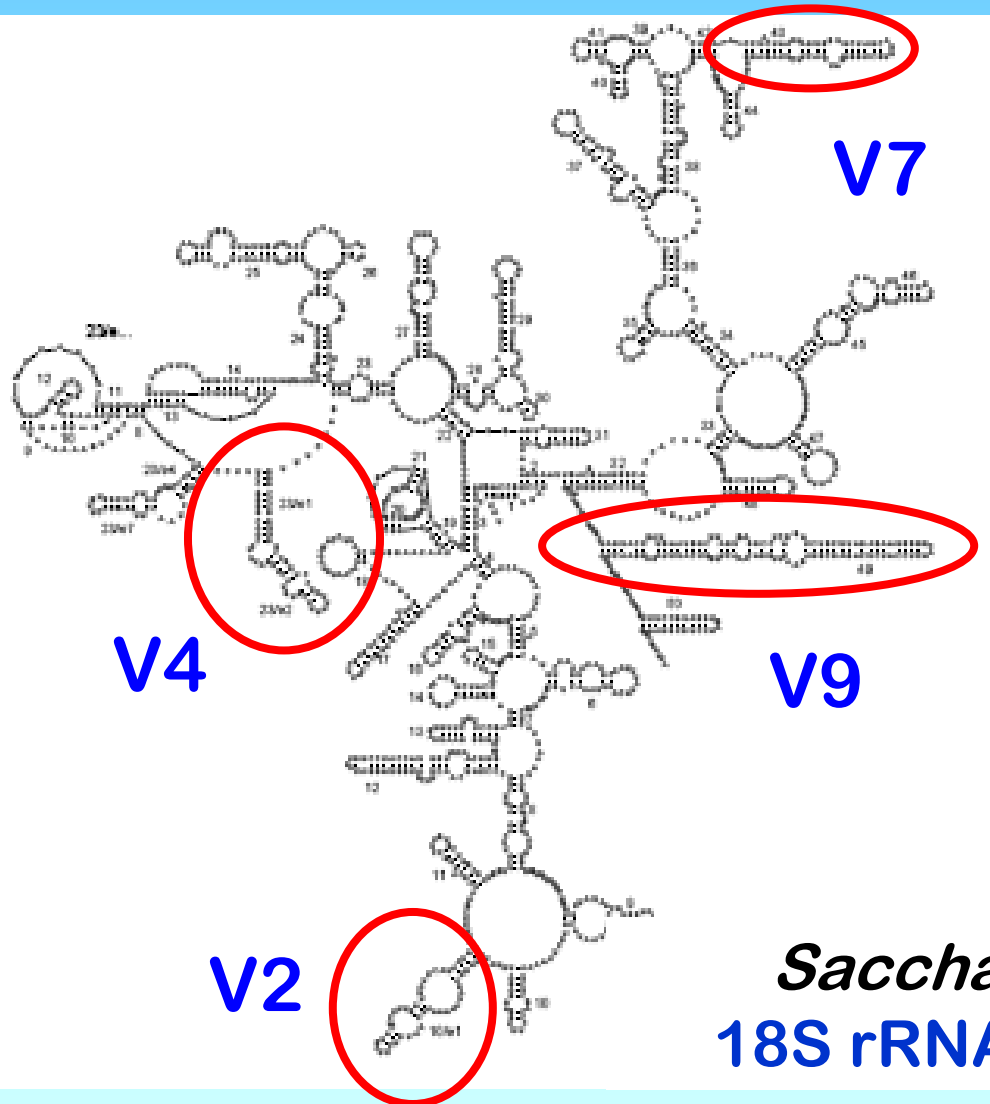
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[5]2D5-Alar-I	0.145	0.142	0.148	0.142	
[6]2D8-Alar-I	0.141	0.137	0.143	0.137	0.002

Conserved core regions alternate with variable expansion segments in the 18S rRNA.



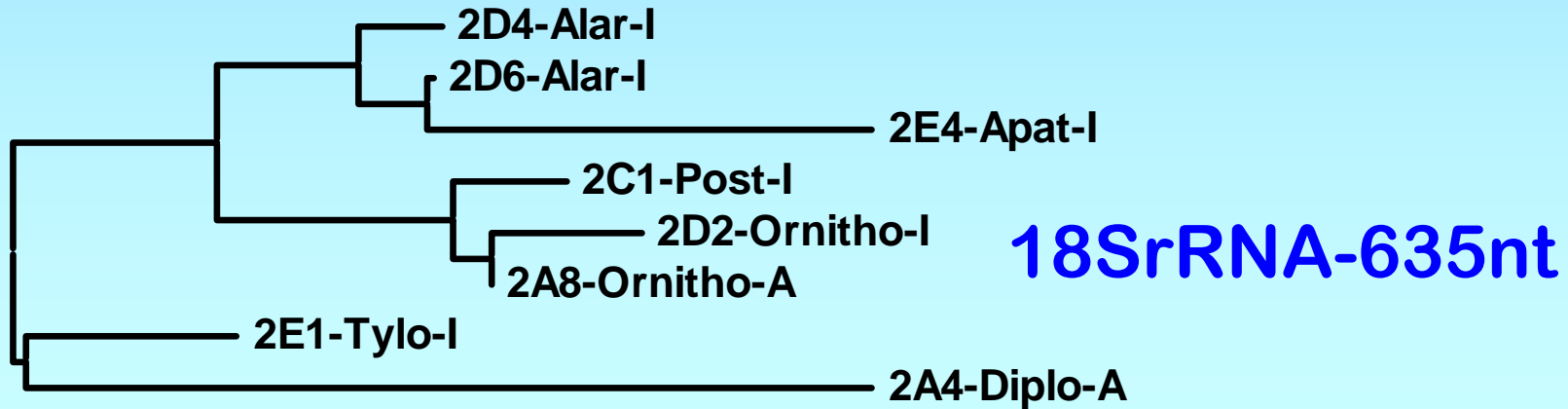
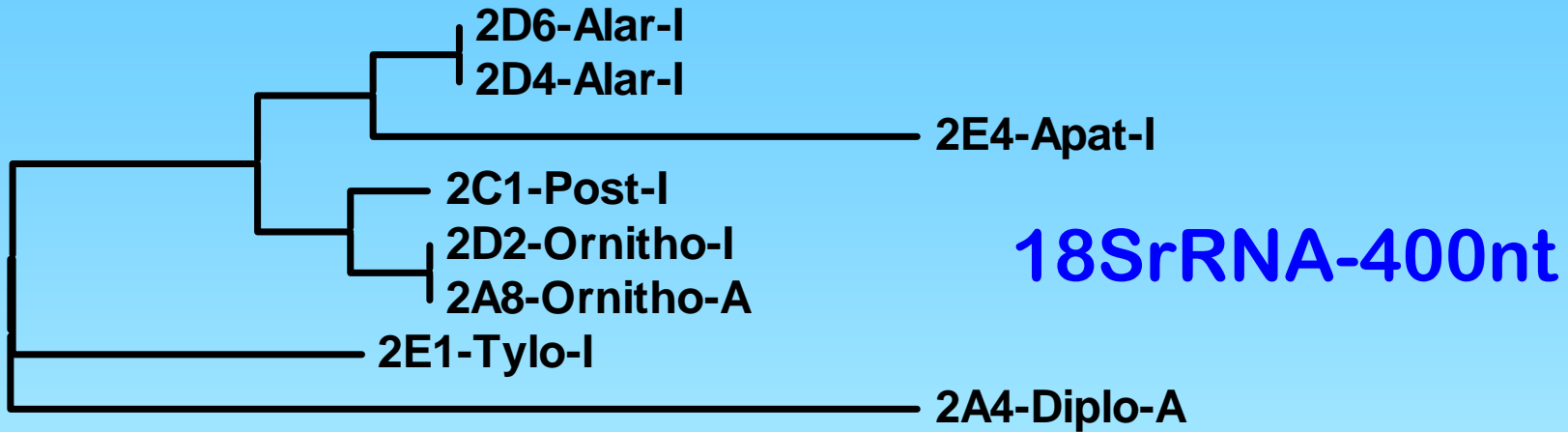
18S rRNA gene sequences have been used to differentiate species in other groups (e.g. Nematodes).

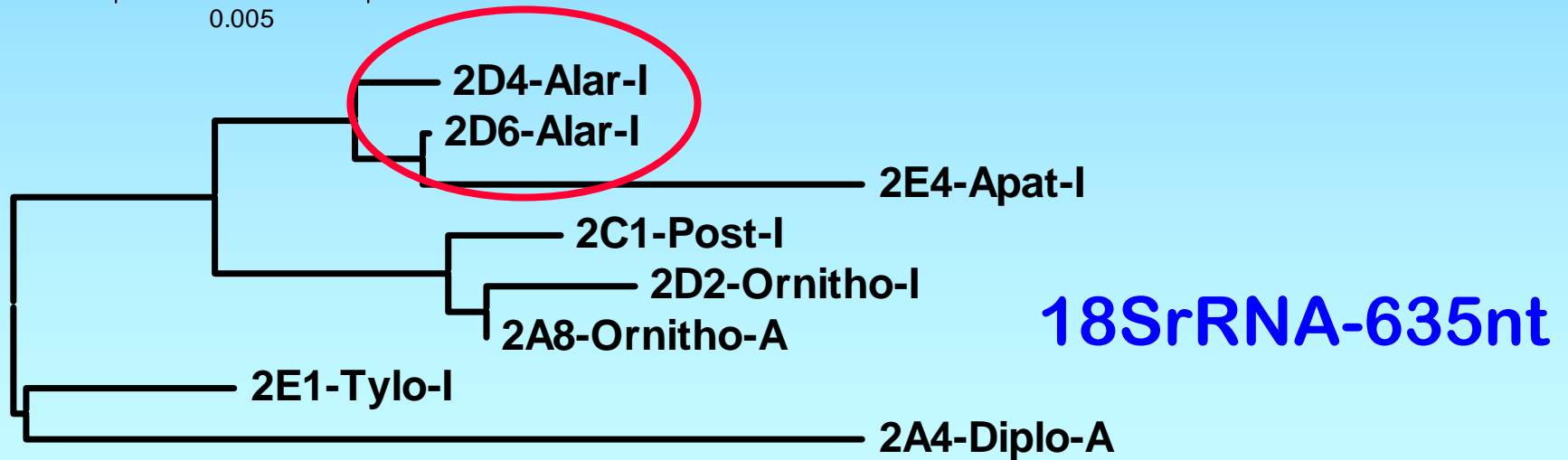
Saccharomyces cerevisiae
18S rRNA secondary structure

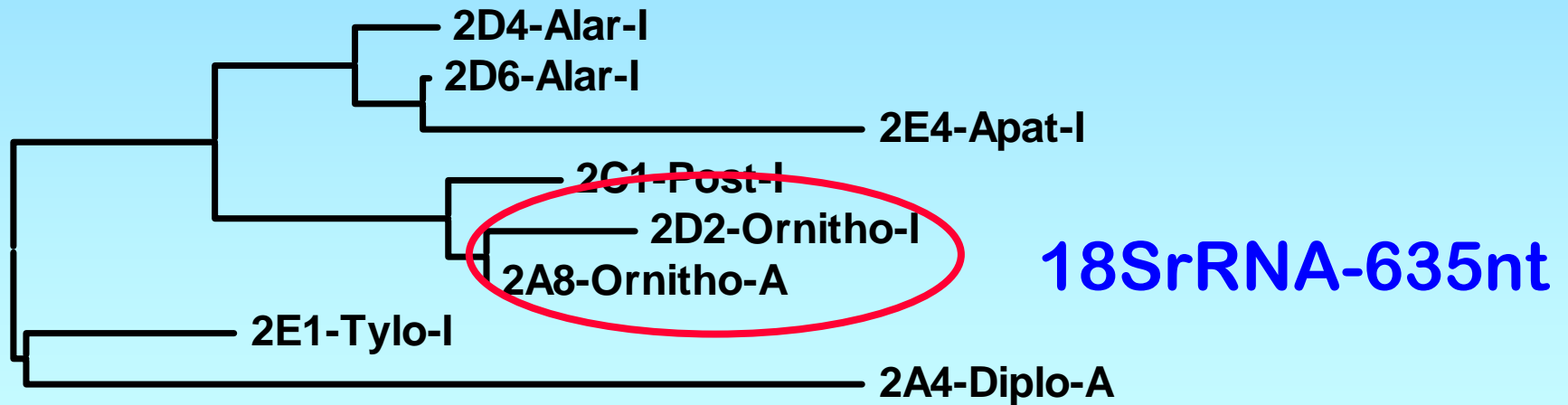
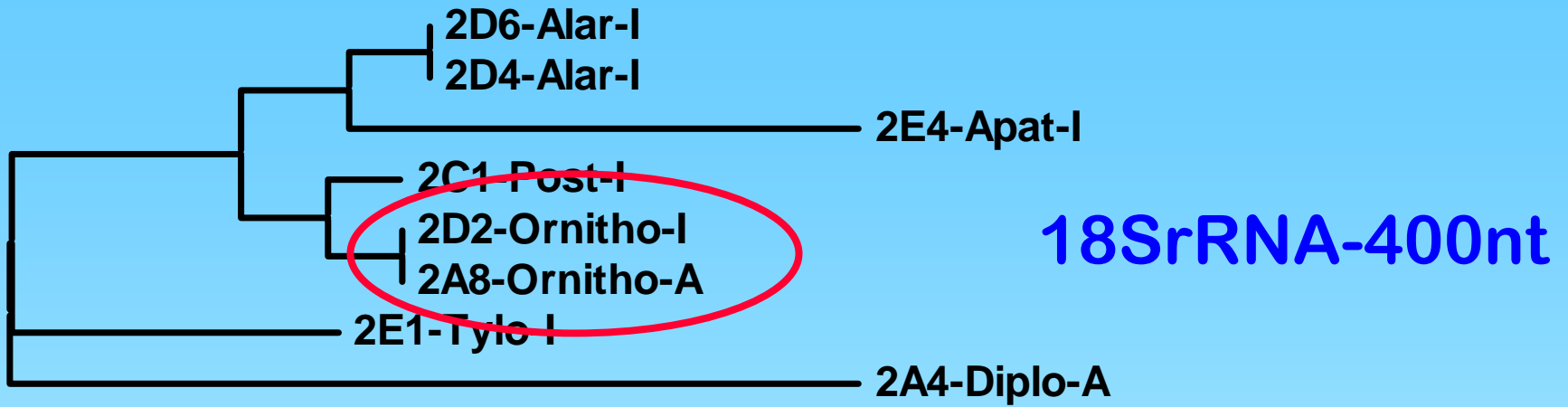
We sequenced the 400 nt 18S rRNA fragment and found that it was able to differentiate genera, but did not identify the groups within species that were evident with cox1 sequences.

We amplified a longer region (635 nt) of the 18S rRNA gene to determine if additional sequence would increase resolution.

The subgroups within genera were recovered with longer 18S rRNA gene sequences.







Flatworm cox1 sequences appear to provide barcode resolution that is similar to that found in other animal taxa.

Amplification success rate with our degenerate cox1 primers is fairly low, especially with larvae of some genera.

Further optimization of PCR reaction conditions may improve amplification success rates.

Quantification of genomic DNA and/or post-PCR cleanup of cox1 fragments may increase sequence quality, but would substantially reduce throughput.

Amplification success rates are high with 18S rRNA gene primers , and the products provide high quality sequence without post-PCR cleanup prior to sequencing.

It may be useful to consider a 2-tiered approach to barcoding flatworms.

- Use 18S (or other) rRNA genes to identify unknown specimens to genus.**
- Develop group-specific cox1 primers to obtain species-level resolution.**

Questions?

18S-635R sequences

	1	2	3	4	5	6	7
[1]2D4-Alar-I							
[2]2D6-Alar-I	0.002						
[3]2E4-Apat-I	0.008	0.006					
[4]2C1-Post-I	0.008	0.008	0.013				
[5]2A8-Ornitho-A	0.006	0.006	0.011	0.002			
[6]2D2-Ornitho-I	0.008	0.008	0.013	0.004	0.002		
[7]2E1-Tylo-I	0.008	0.009	0.015	0.008	0.009	0.011	
[8]2A4-Diplo-A	0.017	0.015	0.019	0.017	0.019	0.021	0.013

18S-9F sequences

	1	2	3	4	5	6	7
[1]2E4-Apat-I							
[2]2D6-Alar-I	0.012						
[3]2D4-Alar-I	0.012	0.000					
[4]2A4-Diplo-A	0.029	0.026	0.026				
[5]2D2-Ornitho-I	0.015	0.006	0.006	0.026			
[6]2A8-Ornitho-A	0.015	0.006	0.006	0.026	0.000		
[7]2C1-Post-I	0.017	0.009	0.009	0.023	0.003	0.003	
[8]2E1-Tylo-I	0.026	0.014	0.014	0.023	0.014	0.014	0.012