Did the surgeon give hepatitis C to his patient?

In a recent issue of the Journal of Medical Virology, R. Stephan Ross and colleagues (2002) report the story of a German surgeon with a viral infection. In July of 2000, the surgeon notified his hospital that he had contracted Hepatitis C Virus (HCV). HCV infects the liver, and is spread by contact with the blood of an infected person. Although many infected individuals show no symptoms, some patients suffer serious liver damage.

The surgeon's specialty was emergency orthopedic surgery. A typical case might involve repairing bones and joints badly damaged in a car wreck. Orthopedic surgery requires a combination of physical

strength and carpentry skill. It involves quick but precise work with saws, hammers, drills, pins, and screws. To a lay spectator, it can appear both violent and bloody. It is not unusual for an orthopedic surgeon, even an unusually careful one, to cut his or her fingers while working inside a patient.

Among the hospital's concerns upon learning that the surgeon had heptatis C was whether he had accidentally passed the infection to any of his patients. The hospital performed blood tests on 207 patients, three of which tested positive for HCV. Among these three, one was known to have been infected before his surgery, and another had a viral strain obviously unrelated to the surgeon's. The last patient, however, had a strain of HCV belonging to the same subtype as the surgeon's. This patient thus presented an open question: Did the patient get HCV from the surgeon, or did he get it from someone else?

We can answer this question by reconstructing an evolutionary tree. This tutorial, and the application ForensicEA Lite, will help you develop the evolutionary logic needed to reconstruct trees from genetic data, and it will teach you one method for doing so. At the end we'll give you data from the paper by Ross and colleagues. You can draw your own conclusions about the German orthopedic surgeon and his patient.

Part 1: Evolution within individual patients

The first step toward developing the tools we need to trace chains of transmission is to recognize that a viral infection is a population of individual virus particles (Figure 1). The infection may be started by one or a few particles that invade the patient's body. But soon the invaders begin to reproduce, establishing a large population. When mutations

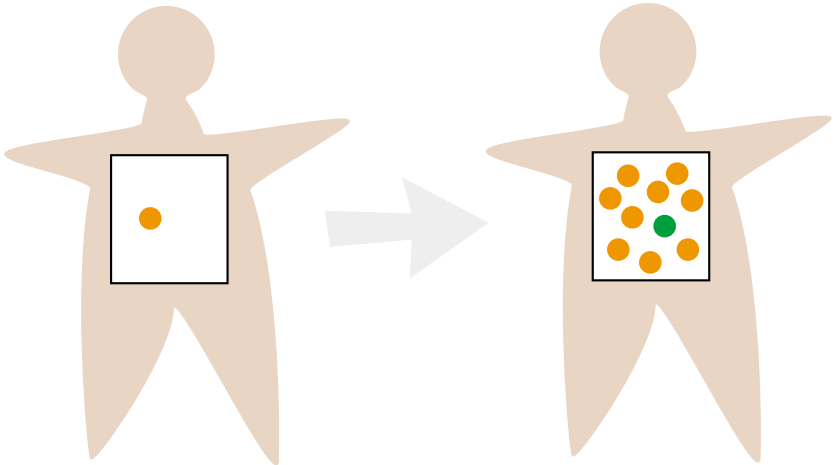


Figure 1 **A viral infection is a population of virus particles.** One or a few virions invade the patient to initiate an infection. Once inside, the virions reproduce, establishing a population. Mutations that occur during viral replication introduce genetic variation. The green virion on the right is a mutant. The population of virions can now evolve.

occur during viral reproduction, the population becomes genetically variable. The population of virus particles can now evolve.

In thinking about how a population of virus particles might evolve, we will imagine that it does so under selection by the host's immune system. (This assumption is will be true for parts of the viral genome that code for proteins recognized and attacked by the immune system. It will not be true for the entire viral genome.) To see how virual populations might evolve under selection by the host's immune system, we will examine change over time in the composition of a simple model population.

To see the model in action, launch the application *ForensicEA Lite*. After the advertisement for *Evolutionary Analysis* disappears, you will see a window titled *Divergence* (Figure 2). The box on the upper left contains our population of virus particles, living inside a patient. We will

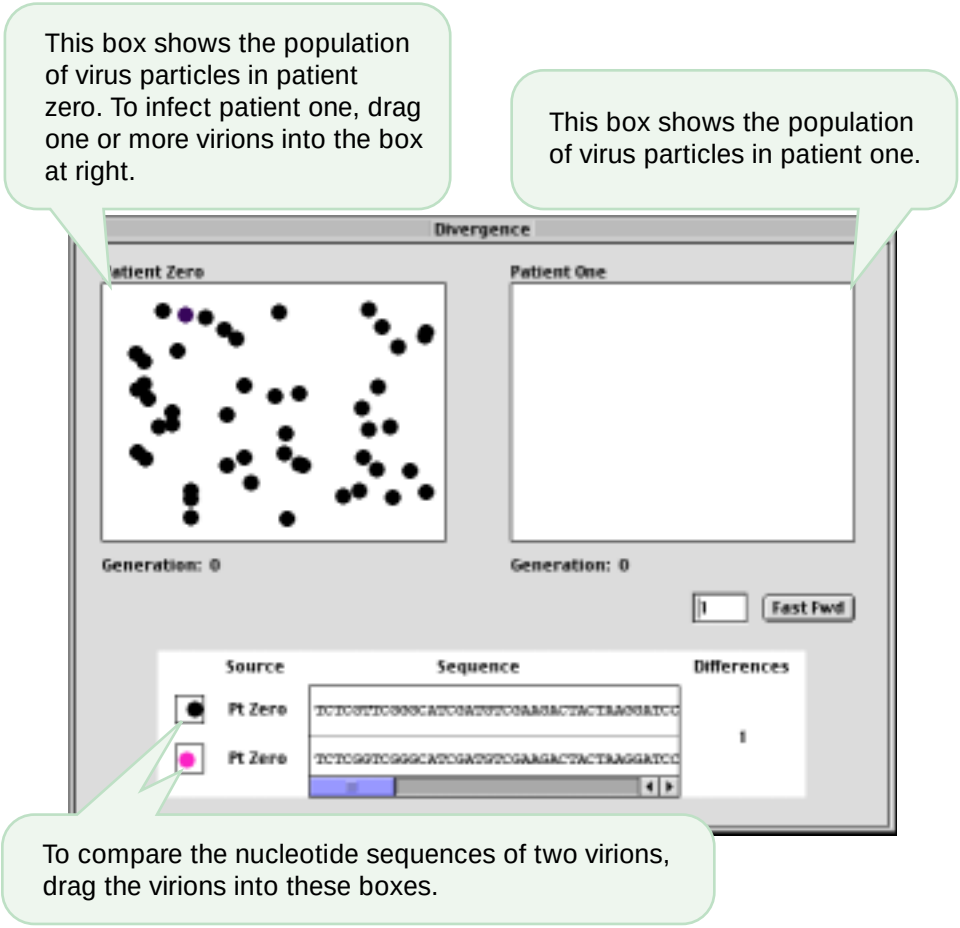


Figure 2 ***ForensicEA Lite's Divergence*** **window.**

call this patient Patient Zero, because he or she will be the patient from which our epidemic starts. To get a closer look at an individual virion inside the patient, click on the virion and hold the mouse button down. A window will pop up showing you a picture of the virion, plus the nucleotide sequence from a stretch of its genome that is 100 base pairs long. (The reader may notice that the nucleotide sequence is written in DNA bases. Although the hepatitis C virus is an RNA virus, we have chosen to represent its genome as a cDNA copy made for sequencing purposes.)

Virus particles with the same color are genetically identical to each other. Note that most, if not all, of the virions in our population are black. These are genetically identical to the virion that initiated our patient's infection. You may see a few virions of different colors. These are mutants that differ from the founder in one or more nucleotides.

You can compare the sequences of two virions by dragging them to the small boxes on the lower left. When you drag virions to these boxes, *ForensicEA Lite* displays the identity of the patient each came from, the nucleotide sequence of each, and the number of differences between the nucleotide sequences.

Drag a virion from Patient Zero to the first small box. Leave it there for the rest of this simulation, so you can use it as a standard of comparison for virions you will collect later.

Reproduction in our model works as follows. Every individual has a chance to replicate itself. Each generation, when it is time for the virions to reproduce, *ForensicEA Lite* picks a virion at random and copies it to make the first offspring. The program then picks another virion at random and copies it to make the second offspring. It repeats this process until we have fifty offspring. Some virions may be lucky and get copied

more than once. Other virions may be unlucky and never get copied at all. Once we have made 50 offspring, all the adult virions die. Then the offspring mature and get a chance to reproduce themselves. A counter below each patient keeps track of the number of generations that have passed since the infection started. Click on the *Fast Fwd* button now to see a new generation of virions. Click it again to see another new generation.

That's nearly all there is to our model. The virions are born, get their chance to reproduce, then die. At this point, you might expect that the population will not evolve at all. There is little or no variation, and, so far, no selection. Variation and selection are necessary ingredients for adaptive evolution.

To generate variation, the model incorporates mutation. As we have already seen, each virus particle has a genome, represented by a piece of cDNA 100 nucleotides long. Every time an adult gets copied to make an offspring, its genome gets copied too. But the copying is not perfect. Occasionally an A is subsituted for a T, or a T for a G, and so on. These mistakes, or mutations, add genetic variation to our population. When a mutation creates a new nucleotide sequence, the virion containing it gets a new color. Watch closely as you click through a few more generations. Occassionally you will see new mutants, with unique colors, appear among the virions in the population.

To incorporate selection by the host's immune system, we imagine that the immune system has learned to recognize the proteins encoded by the virions that were present in previous generations. It has not, however, learned to recognize the proteins encoded by new mutants. We therefore give new mutants a somewhat better chance of reproducing than the rest of the virons in the population.

Note that our model also incorporates chance. Just by luck, some genotypes may reproduce more often than others. These will become more common in the population. Also just by luck, other genotypes may reproduce less often than others. These will become rare, and may disappear altogether. In other words, there are two mechanisms of evolution at work in the population: natural selection and genetic drift.

Make sure you have saved a virion in the upper sequence comparison box. What we want to do now is run the simulation for several hundred generations, sampling a nucleotide's sequence every 50 to 100 generations along the way, and comparing the later sequences to the first one.

Here is a trick to make the simulation run faster. Enter a number, say 25 or 50, in the small text box to the left of the *Fast Fwd* button. Now click the *Fast Fwd* button itself. The simulation now automatically runs for the specified number of generations.

When the simulation stops, pick a virion at random and drag it to the lower sequence comparison box. (A good way to pick a random virion is to simply take the one that landed closest to the lower right corner of the Patient Zero box.) In the table under question 1 on your worksheet, record the generation in which you sampled the new virion, and the number of sequence differences between the new virion and the reference virion. Click *Fast Fwd* again, sample another virion and compare it to the first, and so on. Your goal is to gather data spanning at least 700 generations, recording 10 to 20 sequence differences along the way.

Once you have your 10 or 20 sequence differences spanning several hundred generations, draw a scatterplot showing the number of sequence differences between the present viral genome and the original one (y-axis) as a function of the number of generations that have

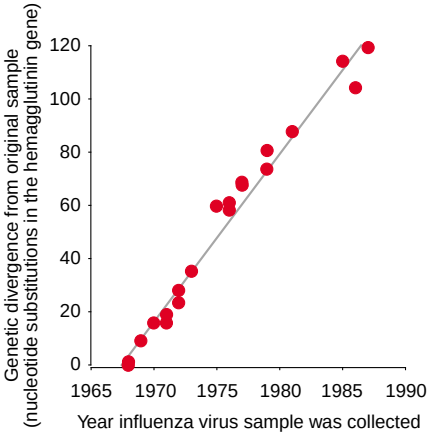


Figure 3 **Evolution of flu viruses** This graph shows sequence divergence as a function of time for flu virus samples preserved between 1968 and 1987.From Fitch et al. (1991).

passed (x-axis). It should look something like the plot for flu viruses shown at left.

Now go to the *File* menu and select *Reset...*. Click the *Okay* button in the window that appears. Repeat the exercise you have just completed.

Think about what happened in your two experiments. What was similar between them? What was different? What generalizations can you make about how populations evolve in this model? If someone gave you frozen samples of virions from your patient, could you make an educated guess as to how far apart in time the samples were collected? Why might an evolutionary biologist think of the graphs you have prepared as molecular clocks?

[For further investigation: You may have noticed that the *Reset* dialog box lets you change the population size, and it lets you change how selection acts on the new mutations that appear. You may want to do some experiments on your own to see how population size, and the pattern of selection, affect the rate at which sequence changes accumulate in populations. If you experiment with the effect of population size on the rate of neutral evolution, be aware that chance can play a large role in any particular run. You will have to run the simulation several times at each of several different population sizes to get a good sense of whether or not population size matters.]

Part 2: Divergence between patients

Having examined sequence evolution in our population of virions in some detail, we can consider what will happen when our patient infects another individual. That is, when one or a few virions move from Patient Zero to Patient One, establishing a new population. The original population and the new one will both continue to evolve. Will they follow similar paths, and thus remain similar in genetic composition? Or will they become steadily more distinct?

Make sure you are in *ForensicEA Lite's Divergence* window. If you are starting a new simulation, use the *Fast Fwd* button to let the simulation run in this patient for 100 generations. This will allow the population to accumulate genetic variation representative of a well-established infection.

Infect Patient One by dragging one or a few virions from Patient Zero into the large box at upper right. Determine the number of nucleotide differences between a randomly chosen virion from Patient Zero and a randomly chosen virion from Patient One by dragging the virions to the small boxes at lower left. (If you have only one virion in Patient One, you can drag it back after you have noted the number of sequence differences.) Record the number of differences in the table on your worksheet.

Now fast forward the simulation for 50 generations. Again sample a randomly chosen virion from each population and record the number of sequence differences. Continue fast forwarding and collecting data until you have accumulated at least 10 measures of sequence difference spanning at least 500 generations.

Plot a graph on your worksheet showing the number of differences between DNA sequences versus the number of generations that have passed since the second population was established from the first. If you had sequences of virions from two infected individuals, could you make a reasonably accurate guess about how long it has been since the virus populations in the two patients shared a common ancestor? That is, could you estimate how far in the past the two patients were connected in the chain of transmission?

[For further investigation: Use the *Reset*... command under the *File* menu to start a new simulation. Experiment with the number of virions transfered to the new patient to start the new infection. Do the populations diverge at an appreciably different rate if you transfer 5, or 10, or 25 virions instead of just one? Is there any effect of population size on the rate at which the populations diverge? Does the rate of divergence depend on whether mutations are neutral, beneficial, or deleterious? How often do you need to transfer individuals between two populations, and how many individuals do you need to transfer, to prevent the populations from diverging?]

Part 3: Evolutionary trees

We have looked at how a viral population evolves within an individual patient, and at how populations in different patients diverge. We are now ready to think about how we might use nucleotide sequences to reconstruct evolutionary history, and to determine whether a particular doctor infected a particular patient.

Reading evolutionary trees

Close all windows in *ForensicEA Lite*. Go to the *Simulation* menu and select *Tree*. This opens the window shown in Figure 4.

At the bottom center of the green area, you will see a small white box. This represents the population of virions inside an individual host. As in the previous windows, you can inspect a particular virion's genome by clicking on the virion and holding the mouse button down. And you can compare the sequences of two virions by dagging the virions to the small boxes at lower left.

We will be dealing with several host individuals in this simulation. Naming them will help us keep them straight. Click on the small white text field above the population box, and type "LC 1." This stands for "Local Control 1." Local Control 1 might be a hepatitis C-infected individual who lives in the same town as the doctor and patient we are investigating, but who is not known in advance to be close (or distant) to either doctor or patient in the chain of transmission.

Now click on the *Fast Fwd* button to start the simulation. Let the simulation run for 100 generations or so. Then, while the simulation is running, click on LC 1's population box. On the screen will appear a new population box, representing a new host. *ForensicEA Lite* automat-

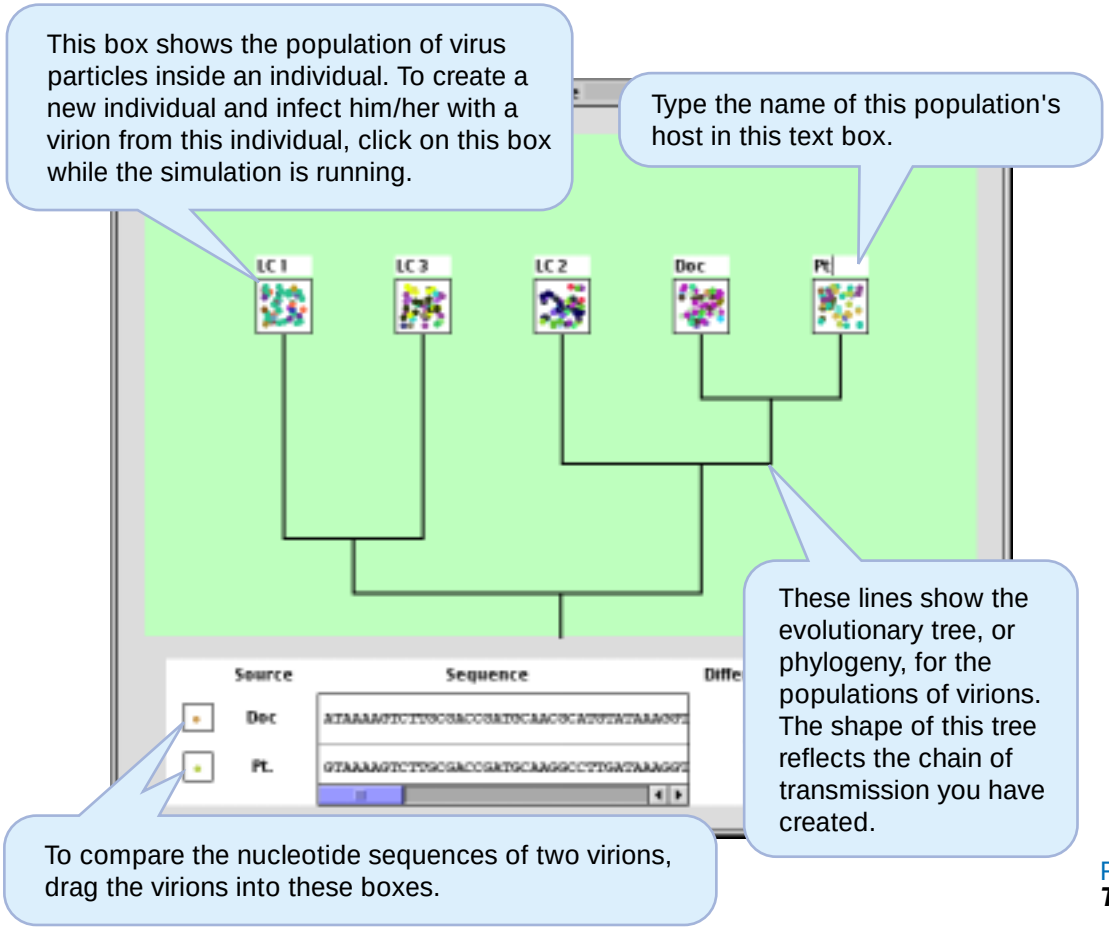


Figure 4 ***ForensicEA*'s *Tree* window.**

ically infects this new host with a virion chosen at random from LC 1's virus population. Name the new host "LC 2." Note that *ForensicEA Lite* is indicating that the virus populations in LC 1 and LC 2 are related to each other by drawing lines below the population boxes that connect them.

Let the simulation run for another 100 generations, then click on LC 1 again. Call the new host "LC 3." How does the evolutionary tree below the population boxes reflect the fact that LC 1 passed HVC to LC 3 more recently than he or she passed it to LC 2?

After another 100 generations, click on LC 2. Label the new host "Doc." This host is the doctor we are investigating, who in this particular scenario picked up HVC from LC 2 (perhaps while performing surgery.) How would the evolutionary tree look if, instead, the doctor had gotten HVC from LC 3? LC 1? A tourist who was visiting from a distant city?

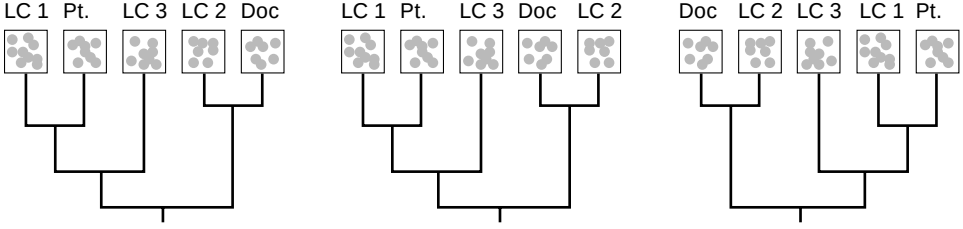
Finally, after an additional 100 generations have passed, click on Doc. Label the new host "Pt." for patient. In this scenario, the doctor has indeed passed HVC to the patient. Let the simulation run for a final 50 generations or so, then click on the *Pause* button. Your evolutionary tree should look like the one in Figure 4 (although your branch lengths might be different).

Examine the evolutionary tree closely. How does it reflect the fact that the patient got HVC from the doctor? What would it have looked like if, instead, the patient had gotten HVC from someone else?

Reset the simulation and run it again, this time creating your own chain of transmission, different from the one in Figure 4. Let the patient pick up the infection from someone other than the doctor. Does the tree look like you thought it would? Make several different trees, reflecting different chains of transmission, until you are sure you could infer how

close two individuals are in the chain of transmission just by looking at the evolutionary tree for their virus populations.

It is also worth thinking about what you *cannot* infer about the chain of transmission just from looking at an evolutionary tree. *ForensicEA Lite* always puts the new host to the right of the host passing the infection, but that was an arbitrary choice. We could have programmed it to put the new host to the left. Or to put the new host on either the right or the left, depending on the toss of a coin. All three of the trees below reflect the same relationships among the hosts's virus populations:



The virus population in LC 1, for example, is more closely related to the population in Patient than either is to the population in LC 3.

However, the trees may or may not reflect exactly the same chain of transmission. For example, LC 2 might have gotten the infection from the doctor, or the doctor might have gotten the infection from LC 2. As a result, we can say with greater confidence what didn't happen—the patient didn't get the infection directly from the doctor—than we can say exactly what did happen.

Are the trees shown here consistent with a scenario in which the doctor was the first individual infected, and thus the ultimate source of all other infections? Are they consistent with a scenario in which the doctor was the last individual infected? The answer to both questions is "yes."

Reconstructing evolutionary trees

Look back at the last evolutionary tree you grew in *ForensicEA Lite*. If the vertical branch lengths on the tree aren't fairly long—if you didn't let at least 50 to 100 generations pass between transmissions—grow another tree in which the branches *are* fairly long.

Your challenge now is to try to reconstruct the evolutionary history of your five virus populations, and infer what you can about the chain of transmission, using only nucleotide sequences sampled from the populations at the branch tips.

This is the same challenge faced by biologists who are trying to unravel the evolutionary relationships among living individuals, populations, or species. The only difference is that you already know the true evolutionary history of your five populations—*ForensicEA Lite* kept track of it for you as the populations evolved. Biologists working with real populations almost never know the true pattern of evolutionary history. The best they can do is make an educated guess about that history based on the available data.

There are many methods for reconstructing evolutionary history based on nucleotide sequences. The details of most methods are mathematically challenging. But the methods have in common the straightforward assumption that the longer it has been since two populations split apart—that is, since they shared a common ancestor—the more

Source of Virion	Nucleotide sequence
Patient 1:	ATATAAGAC? AGCATATTGT ACTCTTAATG GAGCAGAATG GAATAACACT
Patient 2:	?TATAAGACA AGCACATTGT AACCTTAGTA GA?CAGA?TG GAATAA?ACT
Patient 3:	ATATAAGACA AGCACATTGT AACCTTAGTG GAACAGAATG GAGG?AAACT
Patient 8:	CAGAAGGTTG TAGACAAATA CTGGGACAGC TACAGCCATC CCTTCAGACA
Patient 9:	CAGAAGGCTG TAGACAAATA CTGGGACAGC TACAACCGGC CCTTCAGACA

Figure 5 **Nucleotide sequences from the V3 region of the env gene in five HIV virions.** Sequences from GenBank, as noted in Leitner et al. (1996).

genetically different the populations will be. Or to put it another way, the more genetically similar a pair of populations are, the closer they probably are to each other on the tree of life.

The method we will use to reconstruct evolutionary history goes by the acronym UPGMA. This stands for Unweighted Pair Group Method using Arithmetic averages. Never mind what that means. In spite of its name, UPGMA is an intuitively straightforward method. And, while it is not the most powerful method for reconstructing evolutionary trees, it works reasonably well for many data sets.

We will illustrate how UPGMA works by using it to reconstruct the relationships among five HIV virions studied by Thomas Leitner and colleagues (1996). cDNA sequences from a small part of the genome of these five virions appear in Figure 5. The steps in UPGMA are as follows:

1. Determine the genetic distances among all possible pairs of virions. Our measure of genetic distances will simply be the number of single-nucleotide differences between the sequences. The distance between the virion from Patient 1 versus the virion from Patient 2, for

A)

	Pt. 1	Pt. 2	Pt. 3	Pt. 8	Pt. 9
Patient 1	—	5	8	37	38
Patient 2	—	—	3	35	35
Patient 3	—	—	—	38	39
Patient 8	—	—	—	—	4
Patient 9	—	—	—	—	—

B)

C)

	Pt. 1	(Pt. 2-3)	Pt. 8	Pt. 9
Patient 1	—	6.5	37	38
(Patient 2-3)	—	—	36.5	37
Patient 8	—	—	—	4
Patient 9	—	—	—	—

D)

E)

	Pt. 1	(Pt. 2-3)	(Pt. 8-9)
Patient 1	—	6.5	37.5
(Patient 2-3)	—	—	36.75
(Patient 8-9)	—	—	—

F)

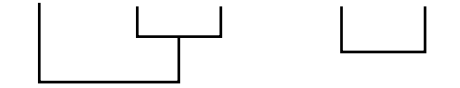


Figure 6 **Using UPGMA to reconstruct the relationships among five HIV virions.** See text for explanation.

example, is 5. Counting up all the pairwise genetic differences produces the numbers in the table in Figure 6A.

2. Scan the table of genetic distances to find the least different pair of virions. In our table, this pair contains the virions from Patient 2 and Patient 3. We will assume these two virions are more closely related to each other than either is to any other virion. Draw a evolutionary tree (a simple one, with just two branches!) and place the virions from Patient 2 and Patient 3 at the branch tips. This tree appears in

Figure 6B. Note that the base of the tree, where the branches leading to the Patient 2 and Patient 3 virions split, is the two virions' common ancestor. We now want to know how this common ancestor is related to the virions from Patients 1, 8, and 9.

3. Revise the table of genetic distances, replacing the Patient 2 and Patient 3 virions with their common ancestor, which we will call the (Patient 2-3) virion. We don't know for certain the genetic distance between the (Patient 2-3) virion the and the Patient 1 virion, but we can estimate it as the average of the Patient 2-to-Patient 1distance and the Patient 3-to-Patient 1 distance. This is (5+8) / 2 = 6.5. Likewise, we can estimate the distance between the (Patient 2-3) virion and the Patient 8 virion as (35+38) / 2 = 36.5, and the distance between the (Patient 2-3) virion and the Patient 9 virion as (35+39) / 2 = 37. We now have the table of distances in Figure 6C.
4. Repeat steps 2 and 3 until the tree is complete. Scanning our new table reveals that the Patient 8 and Patient 9 virions are the least different pair. Placing them on neighboring branch tips gives the tree in Figure 6D. The revised table of genetic distances appears in Figure 6E. Note that our estimate of the genetic distance between (Patient 2-3) and (Patient 8-9) is the average of four genetic distances: Patient 2-to-Patient 8, Patient 2-to-Patient 9, Patient 3-to-Patient 8, and Patient 3-to-Patient 9. The smallest genetic distance now is that between the virions from Patient 1 and (Patient 2-3). Adding this relationship to our evolutinary tree produces the diagram in Figure 6F.

   [Shortly you will use UPGMA to reconstruct your own HVC tree. There is one situation that may arise that we haven't covered. If, when scanning the table of genetic distances, you find a tie for the

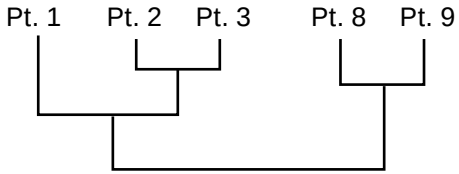


Figure 7 **An estimate of the evolutionary relationships among five HIV virions, reconstructed using UPGMA.**

least different pair, flip a coin to decide which pair you will add to the tree next.]

Our reconstructed evolutionary tree now includes all our HIV virions. All that remains is to connect the two parts of the tree to each other (Figure 7).

How well did UPGMA work? In this particular example, we know the true tree, and the actual chain of transmission. Based on detailed interviews and other evidence, Leitner and colleagues know that Patient 1 was the first of this cluster to become infected with HIV. Patient 1, a Swedish man, later transmitted the infection to two of his female sex partners, Patient 2 and Patient 8. Patient 2 later had a son, Patient 3, to whom she passed the infection. And Patient 8 had a daughter, Patient 9, to whom she passed the infection as well. In this case, the tree we reconstructed with UPGMA is consistent with the true tree. And it is consistent with the known chain of transmission.

Use UPGMA to try to reconstruct the evolutionary tree you produced with *ForensicEA Lite*. Under Question 5 on your worksheet you will find blank tables and spaces to draw your trees.

Your measure of the genetic distance between a pair of hepatitis C virus populations will be the number of single-nucleotide differences between the sequences of virions drawn at random from the popula-

tions. Drag virions to the small boxes at lower left in the *Tree* window, and record the number of differences in Table A.

After you have filled in Table A, complete the UPGMA procedure. Compare the tree you got by using UPGMA to the truth, which was recorded by *ForensicEA Lite* as your populations evolved. (Note that there are many different ways to draw the same tree. The important thing is the pattern of branching among the ancestors. Recall that the trees on page 14 are the same.) How well did UPGMA do at recontructing the truth?

[For further investigation: As we mentioned above, UPGMA is not the most powerful method for reconstructing evolutionary trees. Sometimes it reconstructs the true tree; other times it doesn't. Experiment with using UPGMA to reconstruct a variety of known trees generated in *ForensicEA*. What kinds of trees can UPGMA reconstruct accurately? What kinds of trees does UPGMA do poorly with?]

Part 4: The surgeon and the patient

Figure 8 shows the evolutionary tree R. Stephen Ross and colleagues reconstructed using nucleotide sequences from HCV virions from the German surgeon, is patient, and a variety of controls.

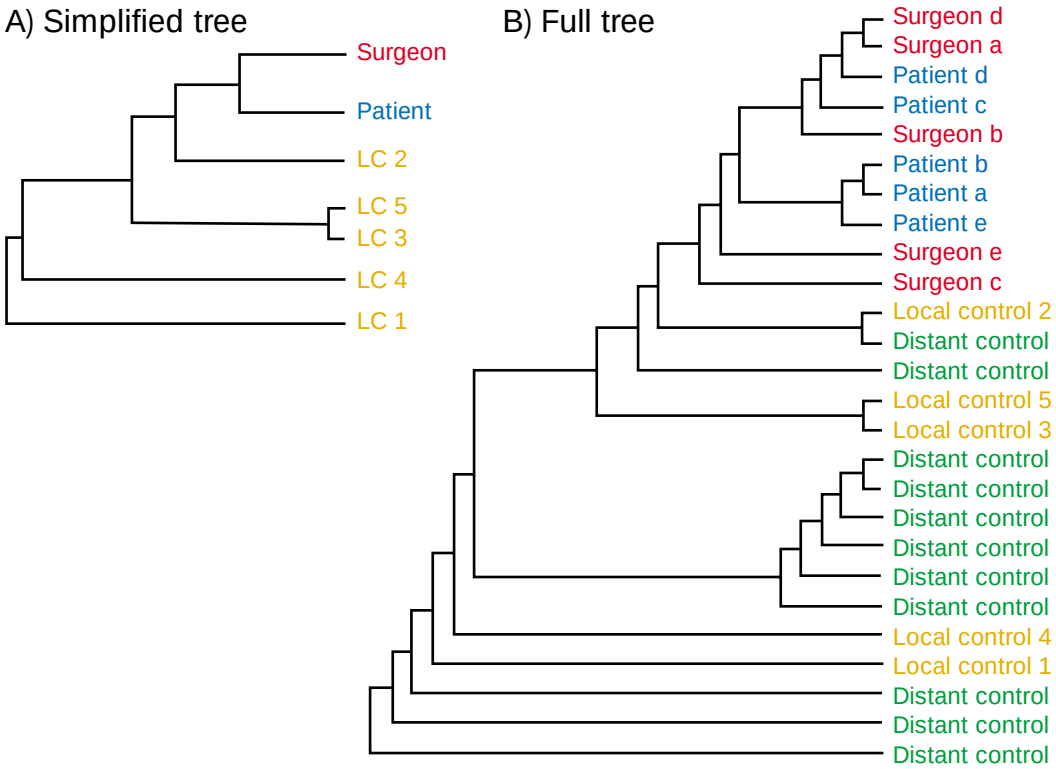


Figure 8 **The reconstructed evolutionary tree for hepatitis C virions from the German surgeon, his patient, and local and distant controls.** Redrawn from Ross et al. (2002).

Look first at the simplified version on the left. This tree includes a single virion each from the surgeon, the patient, and five local controls. Is the tree consistent with the hypothesis that the surgeon passed HVC to the patient? What other interpretations are possible? What other hypotheses can you rule out?

Now look at the full evolutionary tree on the right. This tree includes controls from a variety of cities in addition to the five from the town where the surgeon and patient live. More importantly, it also includes five virions each from the surgeon and the patient. Does the full tree tend to weaken or strengthen the case that the surgeon accidently gave his patient hepatitis? Why?

What do you conclude?

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